

# Flavonoids as regulators of amyloid precursor protein processing

Cox, Carla

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# FLAVONOIDS AS REGULATORS OF AMYLOID PRECURSOR PROTEIN PROCESSING


## CARLA JAYNE COX


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University of Bath

Department of Biology and Biochemistry


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# Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. Amyloid precursor protein (APP) processing and the subsequent generation of amyloid β (Aβ) are central to the pathogenesis of AD, as soluble, oligomeric Aβ peptides are thought to be the toxic species driving disease progression. Flavonoids, a group of dietary polyphenols, have been shown to possess cognitive health benefits. Epidemiological evidence suggests they could play a role in risk reduction in dementia. *In vitro* and *in vivo* reports suggest flavonoids can modulate APP metabolism and Aβ production, although the most effective compounds and the underlying mechanism of action remain unclear. This study identified select flavonoids that were able to reduce amyloidogenic processing in primary cortical neurons at physiologically relevant concentrations. An APP-Gal4 gene reporter assay was characterised for identification of modulators of APP processing in primary neurons. It was tested under physiological conditions, in a pro-oxidant environment or against pathological levels of Aβ production through introduction of a known familial AD (FAD) causing mutation of APP (K595N/M596L). Using this system, five flavonoids were identified that inhibited amyloidogenic APP processing at 100 nM, these were: fisetin, pelargonidin, sinensetin, (-) epicatechin and epigallocatechin. Due to known bioavailability, the catechin family were investigated further and epigallocatechin and (-) epicatechin were confirmed as potent (low nanomolar) inhibitors of amyloidogenic processing. Investigation into the mechanism of action by (-) epicatechin suggested it was likely through indirect BACE1 activity inhibition, independent of BACE1 expression. In addition to activity at APP processing (-) epicatechin also induced extracellular signal-regulated kinase (ERK) activation and transcriptional activity. Investigation of other lead flavonoids also identified fisetin as an inhibitor of ERK-dependent transcriptional activity. This study supports the hypothesis that flavonoids are modulators of APP processing and that they could offer a potential therapy for the prophylaxis of AD.

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# List of Abbreviations

| | |
|---|---|
| (-) EC | (-) Epicatechin |
| 4-HNE | Aldehyde 4-hyroxynonenal |
| 5-HT | 5-Hydroxytryptamine |
| | |
| Aβ | Amyloid β |
| ACE | Angiotensin converting enzyme |
| Ach | Acetylcholine |
| AchE | Acetylcholinesterase |
| AD | Alzheimer's disease |
| ADAM | A disintegrin and metalloproteinase |
| AGE | Advanced glycation end products |
| AICD | Amyloid intracellular domain |
| AMPA | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AO | Antioxidant |
| APH | Anterior pharynx defective 1 |
| APLP | Amyloid precursor like protein |
| ApoE | Apolipoprotein E |
| APP | Amyloid precursor protein |
| APS | Ammonium persulphate |
| ATP | Adenosine triphosphate |
| | |
| BACE | β amyloid converting enzyme |
| BAD | Bcl-2-associated death promoter |
| BDNF | Brain derived neurotrophic factor |
| BPSD | Behavioural and psychological symptoms of dementia |
| | |
| c-Abl | Mammalian Abelson murine leukaemia viral oncogene homolog |
| $Ca^{2+}$ | Calcium |

| | |
|---|---|
| CAT | Chloramphenicol acetyltransferase |
| Cdk5 | Cyclin-dependent kinase 5 |
| cDNA | Complementary deoxyribonucleic acid |
| ChAT | Choline acetyltransferase |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| CR1 | Complement receptor 1 |
| CRE | Cyclic AMP response element |
| CREB | CRE-binding protein |
| CSF | Cerebrospinal fluid |
| CTF | C-terminal fragment |
| | |
| DCC | Deleted in colorectal cancer |
| DIV | Days *in vitro* |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| DR6 | Death receptor 6 |
| | |
| ECE | Endothelin converting enzyme |
| ECG | Epicatechin gallate |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EGC | Epigallocatechin |
| EGCG | Epigallocatechin gallate |
| EGF | Epidermal growth factor |
| Egr1 | Early growth response gene 1 |
| ELISA | Enzyme-linked immunosorbent assay |
| EOAD | Early onset AD |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinase |

| | |
|---|---|
| FAD | Familial AD |
| FCS | Foetal calf serum |
| FDA | Food and drug administration |
| FRET | Fluorescence resonance energy transfer |
| GABA | γ-Amino butyric acid |
| GFAP | Glial fibrillary acidic protein |
| GGA | Golgi-localised γ-ear-containing ARF-binding protein |
| GSAP | Gamma secretase activating protein |
| GSI | Gamma secretase inhibitor |
| GSM | Gamma secretase modulator |
| GSPE | Grape seed polyphenolic extract |
| GTP | Guanosine triphosphate |
| GWAS | Genome wide association study |
| $H_2O_2$ | Hydrogen peroxide |
| HEK293T | Human embryonic kidney 293 SV40 Large T antigen |
| HIF1 | Hypoxia inducible factor 1 |
| HO-1 | Heme oxygenase 1 |
| HRP | Horseradish peroxidase |
| HSV | Herpes simplex virus |
| iCLiP | Intramembrane cleavage protease |
| IDE | Insulin degrading enzyme |
| IGF | Insulin-like growth factor |
| ISF | Interstitial fluid |
| JIP | JNK interacting proteins |
| JNK | C-Jun N-terminal kinase |

| | |
|---|---|
| KIF17 | Kinesin superfamily motor protein 17 |
| KPI | Kunitz type serine protease inhibitor domain |
| LB | Luria Bertani |
| LDL | Low density lipoprotein |
| LOAD | Late onset AD |
| LRP1 | Lipoprotein receptor related protein 1 |
| LTP | Long term potentiation |
| | |
| MAP | Microtubule associated protein |
| MAPK | Mitogen activated protein kinase |
| MDCK | Madin-Darby canine kidney cells |
| MEK | Mitogen activated ERK kinase |
| $Mg^{2+}$ | Magnesium |
| MINT | Munc interacting protein |
| MMP | Matrix metalloproteinase |
| MMSE | Mini mental state examination |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger ribonucleic acid |
| MWM | Morris water maze |
| | |
| NFT | Neurofibrillary tangle |
| NFκB | Nuclear factor kappa-light-chain-enhancers of activated B cells |
| NICD | Notch intracellular domain |
| NIH | National institute of health |
| NMDA | N-Methyl-D-aspartic acid |
| | |
| O/N | Overnight |
| OD | Optical density |
| | |
| PACAP | Pituitary adenylate cyclase-activating polypeptide |
| Paquid | Personnes âgées quid |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD | Parkinson's disease |
| PDGF | Platelet derived growth factor |
| PEN2 | presenilin enhancer 2 |
| pfu | Plaque forming units |
| PI3K | Phosphatidylinositol 3-kinase |
| PICALM | Phosphatidylinositol binding clathrin assembly |
| PKC | Protein kinase C |
| PMA | Phorbol myristate ester |
| PNS | Peripheral nervous system |
| PS | Presenilin |
| PSD | Post-synaptic density |
| PSEN | Presenilin |
| PTB | Phosphotyrosine binding |
| | |
| RECK | Reversion-inducing cysteine-rich protein with Kazal motifs |
| RIP | Regulated intramembrane proteolysis |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| | |
| SAP97 | Synapse associated protein 97 |
| sAPPα | Soluble APP α ectodomain |
| sAPPβ | Soluble APP β ectodomain |
| SDS | Sodium dodecyl (lauryl) sulphate |
| SEZ6L1 | Seizure protein 6 like 1 |
| SNP | Single nucleotide polymorphism |
| SOC | Super optimal broth with catabolite repression |
| SOD1 | Superoxide dismustase |
| SORLA | Sorting-related receptor with A-type repeats |

| | |
|---|---|
| TASTPM | Double-mutant human APPswe×PS1.M146V transgenic mice |
| TBE | Tris/Borate/EDTA |
| TBS | Tris buffered saline |
| TBS-T | TBS + 0.1% Tween 20 |
| Tg2576 | Single mutant human APPswe transgenic mice |
| TGN | Trans-golgi network |
| TIMP | Tissue inhibitors of metalloproteases |
| Tip60 | Tat interacting protein 60 |
| TK | Thymidine kinase |
| $T_m$ | Melting temperature |
| TMD | Transmembrane domain |
| TMP21 | Transmembrane emp24 domain-containing protein 21 |
| TREM | Triggering receptor expressed on myeloid cells |
| TrkB | Tropomyosin receptor kinase B |
| UAS | Upstream activation sequence |
| UV | Ultraviolet |
| VAMP2 | Vesicle-associated membrane protein 2 |
| WT | Wild type |

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*For Mr Reginald Oswald Cox*

# Chapter 1

## 1. General Introduction

# 1. Introduction

## 1.1 Alzheimer's disease

AD is the most common form of dementia and is the tenth biggest killer of people over the age of 65. The biggest risk factor for most AD cases is age: there is a 1 in 40 chance of developing dementia at 65 rising to 1 in 3 at 90 years of age (Figure 1.1). Owing to the demographic trend towards an older population, the number of patients with AD is projected to increase from 24 million in 2005 to 81 million sufferers worldwide by 2040 (Ferri et al., 2005).

Currently, AD costs the UK economy £23 billion annually and this is predicted to rise to £50 billion by 2038 (Alzheimer's Research Trust report 2010). The reasons for this are multifactorial, however increases in informal care and social care are the major costs, direct medical costs are much lower due to the lack of treatments available (Wimo et al., 2013). A projected decrease in the proportion of informal care due to societal family structure changes means UK local authorities are predicted to need to increase home care hours by 91% by 2031 (Comas-Herrera et al., 2007).

AD creates physical, mental and social challenges for the individual and their community and these are much harder to measure. Following diagnosis many patients take a fatalistic view and withdraw from society, not only excluding themselves from societal support, but also putting significant psychological, practical and economic burden on caregivers, often family members (Ferri et al., 2005).

Medical practitioners are still poorly informed as to the best health care to be prescribed to AD patients and many remain uncertain as to the clinical features of AD, resulting in missed opportunities for diagnoses. Currently it is estimated that half of AD sufferers have not received a diagnosis and that there is on average 24 months between first symptoms and diagnosis(INSERM Collective Expertise Centre, 2007) .

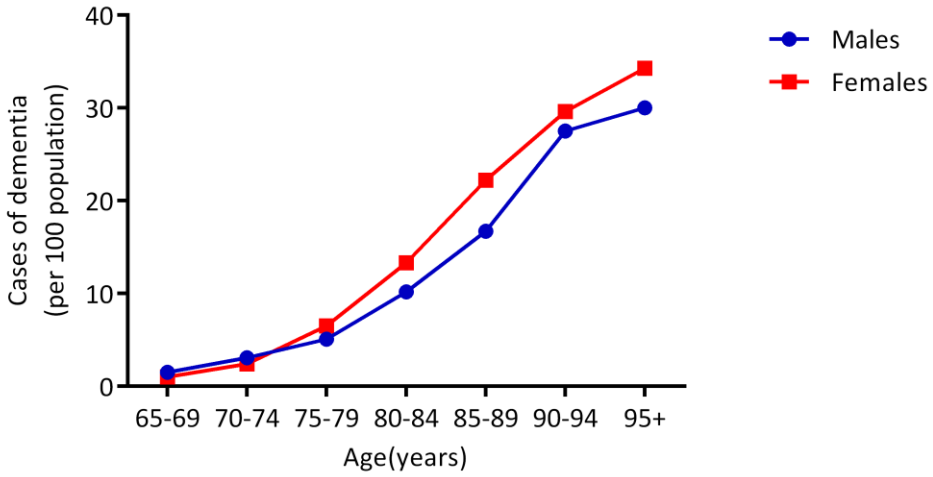



Figure 1.1: **Estimated incidence of dementia in the UK**

(Data taken from Knapp and Prince., 2007).

Once a diagnosis has been made patients can prepare for future decisions such as late stage care and also allows understanding from their community. Due to a lack of approved disease modifying drugs, even those patients that have received a diagnosis only receive symptomatic treatment for their condition; therefore there is a huge unmet medical need for therapies impacting on AD pathology.

# 1.2 Pathology of AD

AD was first described by its pathology over 100 years ago. In 1906, Alois Alzheimer described the case of a woman who had 'peculiar' dementia at 51 years old. He described how her cognitive and behavioural features correlated with the histological findings found on autopsy at death age 55, of 'miliary foci' and neurofibrillary changes in the cerebral cortex (English translation:(Alzheimer et al., 1995)).

## 1.2.1 Symptoms and pathology

The clinical features of AD are progressive loss or decline in memory and cognitive function. These start with episodic memory loss and changes in attention, advancing to language dysfunction, long-term memory problems and at late stages AD patients are mute, non-ambulatory, doubly incontinent and unable to swallow.

Diagnosis of AD is clinicopathological: the patient must present with insidious onset, a history of dementia, and cognitive deficits including amnesia, language deficits, visuospatial deficits or executive dysfunction; diagnosis can only be confirmed at autopsy (McKhann et al., 2011). As well as the cognitive symptoms of AD, there are also behavioural and psychological symptoms of dementia (BPSD) that often affect AD patients. These include depression, psychosis, anxiety and aberrant motor activity (Francis et al., 2010); these often cause significant problems in the care of AD patients, and are the leading cause of admittance of AD patients to residential social care. Symptoms progress over an average time course of 9-10

years, however it is thought the AD pathology begins 10-20 years prior to onset of the clinical symptoms (Holtzman et al., 2011).

Gross visual examination of AD brain shows symmetrical, cerebral atrophy recognised by MRI, with histological lesions in the hippocampus, entorhinal cortex, amygdala and cerebral association cortices whilst sparing sensorimotor cortices (Selkoe and Podlisny, 2002). There are also expansions of the ventricular and sulcal cerebral spinal fluid (CSF) spaces (Ewers et al., 2011) (Figure 1.2(A)). Grey matter loss in patients with AD is 15% more than age matched controls and progresses at a rate of 3-4% per year (Thompson et al., 2003) affecting regions unaffected by age alone (Frisoni et al., 2008). This demonstrates that AD is not simply an acceleration of ageing. Progression of cortical atrophy is tightly associated with progressive cognitive decline, as demonstrated by the correlation between mini mental state examination (MMSE) performance, a global measure of cognition in AD, and grey matter integrity in the entorhinal and parietal cortices (Apostolova et al., 2006). More specific studies have correlated loss of particular cognitive functions to precise areas of cortical atrophy: atrophy in the anterior cingulate and supplementary motor cortices has been associated with apathy in AD patients and the degree of language impairment has been associated with atrophy of the left temporal and parietal cortices (Apostolova et al., 2007, Apostolova et al., 2008).

### 1.2.2 Neuropathology

The pathology of AD follows a stereotypical pathway through progression of the disease. In the early stages of AD, extracellular amyloid deposits occur in the limbic and associated cortices, with temporary, intermittent, alterations to hippocampal synaptic function (Selkoe, 2002). As the disease progresses, intracellular fibrillary tangles develop, initially in the lateral transentorhinal cortex, a critical component in episodic memory (Khan et al., 2014), and amyloid pathology spreads to the striatum and other subcortical regions. Towards end stages of AD there is gross neuronal loss, with all areas of the cortex affected including sensory and motor core fields (Braak and Braak, 1991) (Figure 1.2(A)).

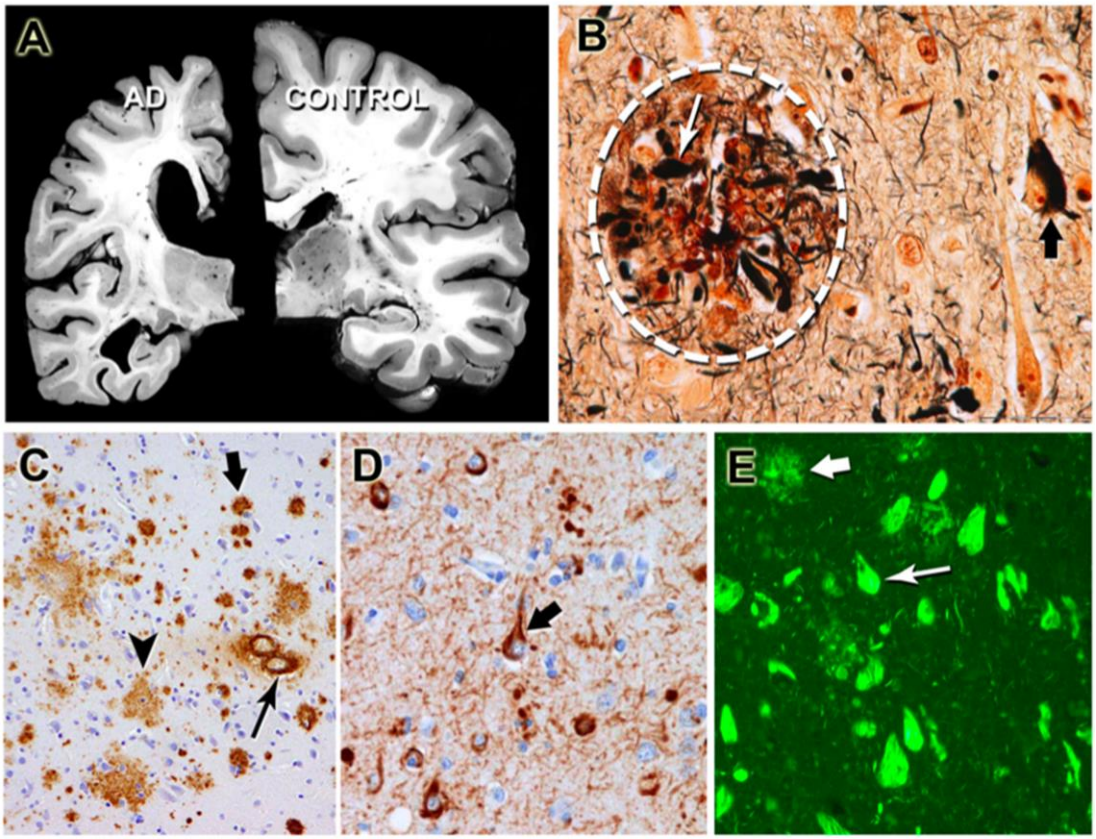



Figure 1.2: **Pathology of AD**

(A) Post-mortem brain sections from AD brain (left) and cognitive normal brain (right) indicated level of cortical atrophy in AD. (B) Photomicrograph using silver stain of amyloid plaque (surrounded by dotted white line), with dystrophic neurites (small arrow) and neurofibrillary tangle (NFT) (large arrow). (C) Immunohistochemical staining of AD brain using anti-Aβ antibody showing diffuse plaques (larger arrow), compact plaques (medium arrow) and cerebral amyloid angiopathy (small arrow). (D) Immunohistochemical staining of AD brain using anti-phospho tau antibody showing hyperphosphorylated tau accumulation in cell bodies (large arrow) and throughout the neuropil (small arrow). (E) Thioflavin-S stain shows β-pleated sheet secondary structure of amyloid plaques (larger arrow) and NFTs (small arrow). (Taken from (Holtzman et al., 2011)).

Cortical atrophy as described above, along with neurotransmitter dysfunction, extracellular Aβ deposition and intracellular tau aggregation are recognised as the hallmarks of AD, each with their own characteristic distribution and progression. Other neuropathological features associated with AD included neuropil threads, microglial activation, and congophilic amyloid angiopathy (Serrano-Pozo et al., 2011).

### *1.2.2.1 Neurotransmitter dysfunction*

Abnormalities in the cholinergic system were amongst the first pathological discoveries in AD brains and the subsequent cholinergic depletion hypothesis was the first theory proposed to explain the aetiology of AD (Bartus et al., 1982). Study of post mortem AD brains demonstrated loss of cholinergic neurons in the basal forebrain and loss of presynaptic markers of cholinergic neurons including choline acetyltransferase (ChAT), acetylcholine (Ach) and acetylcholinesterase (AChE) in the hippocampus and neocortex (Bartus et al., 1982, Whitehouse et al., 1982). These initial suggestions that deficits were due to presynaptic components of cholinergic signalling was questioned following studies showing uncoupling of post-synaptic M1 G-protein signalling correlated well with dementia severity, suggesting a post-synaptic cholinergic deficit (Tsang et al., 2006). This was supported by the disappointing cognitive benefits of AChE inhibitors (AChEIs) as breakdown of M1 mediated intracellular signalling would not be significantly affected by increased levels of Ach in the synapse (Francis et al., 2010).

The cholinergic system is not the only neurotransmitter system affected in AD: deficits in the serotonergic, glutamatergic and GABAergic systems also occur to varying degrees. Extensive serotonergic denervation in post mortem AD brain has been demonstrated although correlation with disease severity remains unclear (Chen et al., 2000). Decreased levels of $5HT_{1A}$ receptors, a pyramidal cell specific subtype of serotonin receptor, has been correlated with MMSE scores suggesting deficits in the serotonergic system contribute to cognitive decline (Kepe et al., 2006).The major loss of serotonergic neurons and associated cell shrinkage, occurs

in the dorsal raphe nucleus, which provides serotonergic projections to the hippocampus, an area severely affected in AD (Zweig et al., 1988, Aletrino et al., 1992).

Glutamatergic pyramidal neurons account for the majority of neuronal cells lost in the cerebral cortex and hippocampus in AD (Francis et al., 2010); the subunit specific dysfunction of N-Methyl-D-aspartic acid (NMDA) receptors in these brain regions suggests that this loss is not due to generalised cortical atrophy and that the glutamatergic system is targeted (Hynd et al., 2004). As well as neuronal loss, dysfunction of the glutamatergic system also contributes to disease progression. Impaired glutamate uptake at the nerve terminal leads to increased background 'noise' at synapses. This causes post-synaptic membranes to become more frequently depolarised, affecting the ability of NMDA receptors (NMDAR) to trigger long term potentiation (LTP) - a cellular signalling pathway that strengthens synapses and is strongly associated with learning and memory (Francis et al., 2010). Furthermore, excessive synaptic glutamate concentrations lead to excitotoxicity in the cell and ultimately neuronal cell death (Mattson, 2003).

Preclinical and clinical studies show that neuronal loss and neurochemical alterations in different transmitter systems are the main factors underlying the cognitive and behavioural symptoms of AD. Loss of particular neuronal populations are strongly correlated with specific cognitive deficits, however the mechanisms and toxic species triggering this widespread cell death are less clear.

#### *1.2.2.2 Neurofibrillary tangles*

Neurofibrillary tangles (NFTs) are intraneuronal aggregates of hyperphosphorylated and mis-folded tau protein (Figure 1.2(D)). Human tau exists in the brain as six isoforms, generated by alternative splicing of its N-terminal domain and microtubule binding domain from a single gene transcript (Goedert et al., 1989). Tau is a major microtubule associating protein (MAP) in neurons, acting to stabilise microtubules and increase microtubule rigidity (Dehmelt and Halpain, 2005). The

protein is unstructured and hydrophilic and in healthy individuals is found primarily in the axonal cytosol (Mandelkow and Mandelkow, 2012).

Phosphorylation of tau tends to weaken microtubule affinity (Illenberger et al., 1998, Mandelkow et al., 2004). Dissociation from microtubules alters the β-sheet structural content of tau, making it prone to self-association and polymerisation to form the paired helical filaments that are present in NFTs (Hanger et al., 1992) (Figure 1.2(E)). The pattern of NFT appearance in AD progression is stereotypical and distinguished into six stages according to the pathological severity (Braak and Braak, 1991). The amount and distribution of NFTs closely correlate to the level and sequence of brain atrophy and associated cognitive decline in AD (Braak and Braak, 1991, Johnson et al., 2012, Khan et al., 2014). They start in the lateral transentorhinal cortex (episodic memory), followed by the prefrontal cortex (executive function), parietal cortex (apraxias), and occipitoparietal cortex (visuospatial deficits), although whether NFT formation is a requirement for neuronal death in AD is still controversial.

### *1.2.2.3 Amyloid plaques*

Amyloid plaques are proteinaceous extracellular deposits resulting from abnormal accumulation of Aβ, a physiological product of APP metabolism. The plaques have a central deposit of Aβ (the core), intimately surrounded by dystrophic neurites and activated microglia and astrocytes (Selkoe, 1991b) (Figure 1.2(B)). Within the plaques, Aβ exists in insoluble forms including fibrils and oligomers with a predominantly β- sheet structure. Although Aβ can be released as a 38-42 amino acid peptide, $A\beta_{42}$ is the predominant form in plaques due to its increased rate of fibrillisation and its increased hydrophobicity. The ratio of $A\beta_{42}$ to the less hydrophobic $A\beta_{40}$ product is now thought to be the driver of fibrillisation, perhaps due to the ability of $A\beta_{40}$ to sequester $A\beta_{42}$ and facilitate its clearance (Kumar-Singh et al., 2006). The Aβ content of the brain is not restricted to that deposited in the characteristic plaques. A large proportion of it is in 'diffuse' structures which do not have β- sheet structure or associated dystrophic neurites; instead the Aβ exists as

granular structures or as fine filaments. Despite this, it is the dense-core plaques that are associated with the synaptic and neuronal loss characteristic of AD (Selkoe and Podlisny, 2002, Serrano-Pozo et al., 2011). In addition to the extracellular deposits of Aβ, almost all AD brains display deposits of fibrillar Aβ in the basement membranes of scattered microvessels, although the significance of these is not clear (Figure 1.2(C)).

Unlike NFTs, the spatiotemporal progression of amyloid plaques does not correlate well with neuronal cell death or cognitive decline. There is also significant dissociation between the brain regions affected by NFTs and amyloid deposition. Although less predictable that NFT progression, staging of amyloid pathology into three stages has been possible. It begins in the isocortex, followed by deposition in isocortical association and limbic areas, and the final stage is characterised by amyloid deposits in primary isocortical areas and subcortical structures (Braak and Braak, 1991, Thal et al., 2002).

Amyloid pathology is evident even at preclinical stages of AD, prior to any evidence of cognitive decline. The appearance of amyloid pathology early in disease progression and the lack of correlation with cognitive function have led to a growing consensus that Aβ is the driver of disease progression leading to increases in tau phosphorylation which initiates pathways involving loss of synaptic transmission that leads to neuronal cell death.

# 1.3 Genetics of AD

## 1.3.1 Familial AD

Once APP had been identified as the precursor to Aβ, genetic studies were undertaken on brains of familial AD (FAD) patients to try and delineate the genetic causes of the disease. AD is generally considered a disease of the elderly, however like the first case described by Alois Alzheimer, a proportion of AD sufferers, currently estimated to be less that 1%, develop the disease before the age of 65.

This form of AD, called early onset AD (EOAD) or FAD, shows autosomal dominant inheritance. To date, more than 32 mutations in the APP gene linked with development of AD have been discovered (Cruts and Van Broeckhoven, 1998) http://www.molgen.ua.ac.be/ADmutations). Select mutations have been taken forward into transgenic rodent models of AD and have been shown to be sufficient to produce pathology associated with AD, including gross neuronal cell death, altered synaptic activity, plaque formation and intracellular tangles (Walsh et al., 2002, Lesné et al., 2006, Meyer-Luehmann et al., 2008) (Table 1.1). Although APP mutations were identified first, the majority of mutations associated with FAD are found in the *presenilin 1* gene on chromosome 14, where over 200 mutations have been reported (Larner, 2013). The *presenilin 1* gene encodes for the catalytic domain of γ-secretase, the enzyme responsible for release of Aβ from the membrane (Kimberly et al., 2000). The remaining mutations identified are located on chromosome 1 in the homologous *presenilin 2* gene.

### *1.3.1.1 APP mutations*

In 1991, a point mutation was identified in a family from London on chromosome 21 (V717I) that segregated with FAD patients (Goate et al., 1991). Following this initial identification, a wave of publications through the 1990's and 2000's reported further mutations (Table 1.1 and Figure 1.5(B)); all clustered around the cleavage sites of the Aβ sequence in APP (Figure 1.5(B)). Like the initial mutant identified, most APP mutants were proximal to the C-terminus of Aβ , these alter APP processing by increasing the proportion of longer, more aggregation prone, Aβ peptides generated (Suzuki et al., 1994, Tamaoka et al., 1994, Eckman et al., 1997), affecting the $A\beta_{40}$:$A\beta_{42}$ ratio.

A further group of mutations cluster around the N-terminal of the Aβ sequence, causing increases in total Aβ levels (Hendriks et al., 1992, Zhou et al., 2011). Cleavage at the N terminus of the Aβ sequence can occur at two distinct sites called the β and β'. Mutations at the N-terminal of Aβ have been suggested to favour the

| Mutation ($APP_{770}$ numbering) | Family Population | Effects on Aβ | References |
|---|---|---|---|
| **K670N/M671L** | Swedish | ↑ Aβ generation<br>altered subcellular activity of BACE1 | (Citron et al., 1992, Mullan et al., 1992, Cai et al., 1993, Haass et al., 1995b) |
| **A673T** | Iceland | ↓ Aβ generation | (Jonsson et al., 2012) |
| **E682K** | Leuven | ↑ Aβ generation<br>shift cleavage from β' to β site. | (Zhou et al., 2011) |
| **A692G** | Flemish | Microvascular Aβ accumulation | (Hendriks et al., 1992) |
| **E693G** | Arctic | ↑ Aβ fibrillogenesis | (Nilsberth et al., 2001) |
| **E693K** | Italian | Microvascular Aβ accumulation | (Bugiani et al., 2010) |
| **E693Q** | Dutch | Microvascular Aβ accumulation | (Van Broeckhoven et al., 1990) |
| **D694N** | Iowa | ↑ Aβ fibrillogenesis | (Grabowski et al., 2001, Van Nostrand et al., 2001) |
| **T714I** | Austrian | ↑ $Aβ_{42}$ production<br>↓ $Aβ_{40}$ production<br>↑ $Aβ_{42:40}$ | (De Jonghe et al., 1998, Kumar-Singh et al., 2000) |
| **V715A** | German | ↑ $Aβ_{42:40}$ | (Cruts et al., 2003) |
| **V715M** | French | ↓Aβ production<br>↑ $Aβ_{42:40}$ | (Ancolio et al., 1999, Campion et al., 1999) |
| **I716V** | Florida | ↑ $Aβ_{42}$ production<br>↑ $Aβ_{42:40}$ | (Eckman et al., 1997) |
| **V717I** | London | ↑ $Aβ_{42}$ production<br>↑ $Aβ_{42:40}$ | (Goate et al., 1991, Naruse et al., 1991, Karlinsky et al., 1992, Tamaoka et al., 1994) |
| **V717P** | unknown | ↑ $Aβ_{42}$ production<br>↑ $Aβ_{42:40}$ | (Murrell et al., 1991) |

Table 1.1: **APP mutations associated with FAD**

Amino acid substitutions of APP that lead to onset of familial AD and their effects on Aβ. A673T is the only mutation shown to be protective

β rather than β' site, this leads to generation of more full length Aβ, such as in the case of the Flemish mutant E682K (Zhou et al., 2011). Perhaps the most well studied mutant of APP is the Swedish, double point mutation of K670N/M671L, located immediately adjacent to the β-cleavage site. This causes 6-8 times more production of Aβ than WT APP due to alterations in the subcellular compartment in which cleavage occurs, promoting Aβ production (Citron et al., 1992, Cai et al., 1993, Citron et al., 1994, Haass et al., 1995b). In contrast to the Swedish and Flemish mutations, a further mutant in this region, the Icelandic A673T reduces amyloidogenic peptide formation by 40% in *in vitro* tests (Jonsson et al., 2012), the first mutant identified that confers Aβ lowering effects.

Another cluster of mutations are located within the Aβ sequence, such as the Arctic (E693G), Iowa (D694N) and Dutch (E693Q) mutations. These are suggested to affect the aggregation properties or the degradation efficiency of the Aβ peptide (Van Broeckhoven et al., 1990, Nilsberth et al., 2001, Van Nostrand et al., 2001)(Table 1.1).

#### *1.3.1.2 PSEN mutations*

PSEN1 mutants show dominant inheritance and are responsible for 70% of FAD cases (Sherrington et al., 1995, Larner, 2013). There are no nonsense mutations identified to date, suggesting the mutations in *Presenilin 1* alter the function of γ-secretase, rather than resulting in a loss of function (Clark et al., 1996). This is in contrast to the observation that *Presenilin 1* mutations are distributed throughout the gene, with no obvious clustering (Figure 1.3). This paradox is hard to align, however it may relate to essential role for presenilins where complete loss of function would be lethal and therefore only mutations leading to partial loss can persist in the population, although further evidence is required to confirm this.

There is broader heterogeneity in phenotypes associated with *PSEN1* mutations compared to *APP* mutations. Individuals with *PSEN1* mutations often display behavioural and psychiatric symptoms not typically seen in individuals with *APP* mutations. These additional phenotypes have been well documented although the

mechanisms are not well understood. For example, the splice mutation ΔE9 leading to frame skipping of exon 9 and an amino acid substitution introducing a cysteine has additional clinical features including spastic paraplegia (Cruts and Van Broeckhoven, 1998). Other mutations also present with additional features such as early language impairment (V272A), myoclonus (L226F) or seizures (H163R and L282R) (Larner, 2013).

Unlike *APP* mutations, the age of onset varies depending on the specific *PSEN* mutant expressed, with a range from 30-60 years old. *PSEN1* mutants reduce γ-secretase activity, reducing the absolute levels of Aβ, but also alter the cleavage activity of the enzyme, favouring production of longer Aβ species, and increasing the $A\beta_{42:40}$ ratio (Bentahir et al., 2006, Bergmans and De Strooper, 2010, Quintero-Monzon et al., 2011) therefore increasing the likelihood of Aβ aggregation.

*PSEN2* mutations were originally identified through 7 families with FAD where linkage to chromosomes 14 and 21 had been excluded (Levy-Lahad et al., 1995a, Levy-Lahad et al., 1995b). *PSEN2* mutations are much less prevalent than *PSEN1* mutations and cause a later age of onset (50-70 years) (Rogaev et al., 1995). Mutations in *PSEN2* occur at amino acids conserved within the presenilin family, suggestive of similar mechanisms to *PSEN1* mutations. This is supported by studies such as that looking at the N141I missense mutation of PSEN2, found in the Volga German kindred, which found increased $A\beta_{42}$ levels in a transgenic mouse from 2 months of age (Levy-Lahad et al., 1995a, Oyama et al., 1998)

### 1.3.2 Genes associated with LOAD

Despite the large number of highly penetrant mutations identified that lead to FAD, these mutations are rare and the majority of AD cases show no clear genetic linkage and present in patients over 65 years old; these are termed ‘sporadic’ or late onset AD (LOAD). In these cases age is the biggest risk factor, however genetic studies have also identified high frequency but low penetrance genetic risk factors, increasing risk by small percentages.

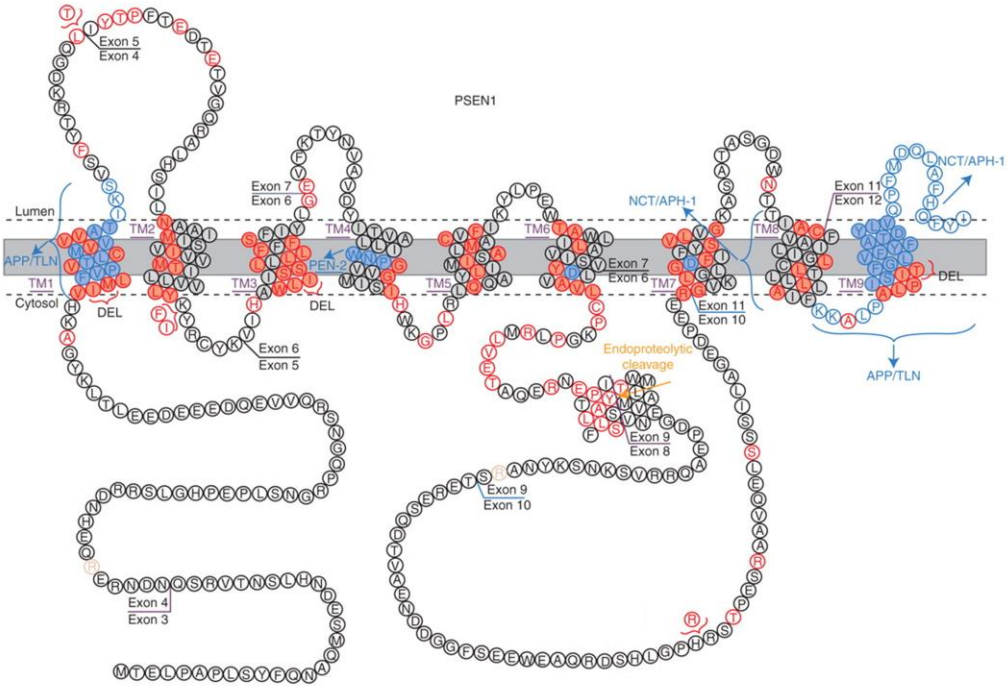



Figure 1.3: **Presenilin mutations**

Amino acid sequence of the 9 TM topology and mutations of PS1 including exon boundaries. FAD- linked mutations are indicated in red. Residues involved in the interaction with amyloid precursor protein (APP), PEN-2, Nicastrin (NCT) and APH1 are indicated in blue. The point of endoproteolytic cleavage is indicated with a yellow arrow. (Taken from (de Strooper et al.,2012)).

### *1.3.2.1 Apoε4*

The most well established genetic risk factor for LOAD, consistently identified in all genome wide association studies (GWAS), is the *apolipoprotein E* (*ApoE*) gene. ApoE, produced by astrocytes and neurons in the central nervous system (CNS), regulates lipid homeostasis through transport of cholesterol to neurons. *ApoE* has a 3 allele haplotype: Apoε2, Apoε3 and Apoε4 differing in the amino acids at residues 112 and 158, affecting the lipid carrying abilities of the protein in a dose dependent manner (Corder et al., 1993, Liu et al., 2013). The ε4 (Arg112, Arg158) allele increases risk between 3.7 (single copy) and 10 fold (two copies) (Suh et al., 2013). Contrary to increased risk with ε4, the ε2 (Cys112, Cys158) allele has been shown to exert protective effects and is associated with healthier aging. The ε3 (Cys112, Arg158) allele appears to be neutral in comparison (Bertram et al., 2010). The different binding properties of the ApoE isoforms to Aβ and tau have been suggested to form the bases of the individual phenotypes. The Apoε4 isoform has been shown to bind Aβ much more rapidly than the other isoforms, leading to increased fibrillisation (Strittmatter et al., 1993, Sanan et al., 1994) and exhibits impaired binding to tau in comparison to Apoε2 and Apoε3, suggesting that the ApoE/tau interaction is protective (Strittmatter et al., 1994).

### *1.3.2.2 Other risk factor genes*

Early genomic studies consistently identified ApoE as the single significant genetic risk factor for AD. Subsequent larger studies have identified risk loci with smaller effect sizes leading to identification of *clusterin,* phosphatidylinositol binding clathrin assembly *(PICALM),* complement receptor 1 (*CR1)* and triggering receptor expressed on myeloid cells 2 (*TREM2)* genes as novel risk genes.

The *Clusterin* gene encodes for the second major apolipoprotein in the brain after ApoE and was identified as a novel susceptibility locus in two independent GWAS studies (Harold et al., 2009, Lambert et al., 2009). Elevated Clusterin expression in the hippocampus and entorhinal cortex of AD brains and presence of Clusterin in

senile plaques provides functional evidence of clusterin involvement in AD pathogenesis (May et al., 1990, Oda et al., 1994). Variants of Clusterin appear to have a positive effect in AD, as it can modify clearance of Aβ at the blood brain barrier and together with Apoε4 suppress Aβ fibrillisation (DeMattos et al., 2004, Bell et al., 2007). Clusterin also plays a role in ameliorating Aβ-mediated activation of an immune response, possibly through masking fibrillar structures from the immune system (Nuutinen et al., 2009).

*PICALM* encodes for the phosphatidylinositol-binding clathrin assembly protein. It was initially identified as a risk locus in one of the same GWAS studies as clusterin (Harold et al., 2009). PICALM is ubiquitously expressed with particularly high expression in neurons. It is involved in clathrin mediated endocytosis, a processing essential for correct lipid and protein trafficking (Kim and Kim, 2001). In neurons, PICALM is essential for Vesicle-associated membrane protein (VAMP2)-mediated neurotransmitter release at the pre-synaptic membrane (Harel et al., 2008). Variants of PICALM in AD have therefore been proposed to cause synaptic perturbations or alterations in APP processing through endocytic pathways. Depletion of PICALM *in vitro* and *in vivo* led to dendritic dystrophy and disrupted secretory transport, and caused selective alterations to $A\beta_{42}$ production, reducing the $A\beta_{42:40}$ ratio (Bushlin et al., 2008, Kanatsu et al., 2014).

*CR1* encodes a type 1 transmembrane glycoprotein. It is the main receptor for complement C3b protein that mediates binding of particles and immune complexes that have activated complement. Both the classical and alternative complement pathways are associated with AD. mRNA levels of components from each pathway are elevated in AD brains and the complement system is activated by both amyloid plaques and NFTs (McGeer et al., 1989, Strohmeyer et al., 2000, McGeer and McGeer, 2001). Chronic inflammation in AD is a consistent event, however whether it is beneficial or a mediator of neurodegeneration is as yet undecided. Therefore whether variants of CR1 are having a positive or detrimental impact remains unclear.

The *TREM2* (triggering receptor expression on myeloid cells 2) gene encodes another type 1 transmembrane protein that triggers activation of an immune

response. TREM2 is expressed on microglial cells throughout the CNS. It stimulates phagocytosis, enhancing cell debris clearance and also suppresses cytokine production and inflammation. Reduced activity of TREM2 variants is central to their pathogenic effects, potentially through reduced clearance of Aβ by phagocytosis or a relief of the block on inflammatory cascades (Frank et al., 2008, Guerreiro et al., 2013).

### *1.3.2.3 ADAM10 mutation*

*ADAM10* encodes for a type 1 transmembrane protein with zinc metalloprotease activity. ADAM10 acts as the major α-secretase activity in neurons that prevents production of Aβ from APP through cleavage within the Aβ sequence (Kuhn et al., 2010). Missense mutations in the prodomain of the ADAM10 gene have been shown to cause LOAD. In contrast to other genes associated with LOAD, genetic association studies of SNPs in the ADAM10 gene led to identification of two mutations that showed high although incomplete penetrance (Kim et al., 2009). The two mutations, Q170H and R181G, located proximal to the prodomain cleavage site, were introduced separately into mice to generate 2 transgenic lines. Both showed reduced ectodomain shedding and attenuated ADAM10 activity and crossing with an APP mutant mouse (Tg2576) increased Aβ levels, plaque load and reactive gliosis. The APP family are particularly vulnerable to changes in ADAM10 activity, as N-cadherin and notch levels were unaffected by the mutations whilst APLP2 showed a similar sensitivity to APP. ADAM10 plays a critical role in neurogenesis through release of sAPPα, which was also severely impaired in the mutant mice (Suh et al., 2013). Studies of sAPPα levels in AD patients carrying these mutations have not been published, however there have been no previous reports of changes in sAPP levels in AD brain therefore the mechanism of these mutants remains unclear (Brinkmalm et al., 2013).

It has been estimated that *APP*, *PSEN1*, *PSEN2* and *ApoE* gene mutations account for less that 30% of the genetic variance in FAD and LOAD (Guerreiro et al., 2012). *Clusterin*, *PICALM*, *CR2* and *TREM*2 are therefore likely to be just the tip of the

iceberg in further genetic risk factors. These examples give an indication of the complexity of AD, and give an indication of the range of cellular pathways involved in AD pathogenesis.

## 1.4 Amyloid cascade hypothesis

An early clue for the cause of AD pathology was the link between trisomy 21 (Down's syndrome) and AD: Down's syndrome patients will invariably develop AD-like symptoms in their fourth or fifth decade (Tanzi et al., 1988), linking chromosome 21 to Alzheimer's-like symptoms. A second disease called cerebral haemorrhage with amyloidosis (Dutch type) was shown to result from a mutation in APP, located on chromosome 21,, suggesting that mutations in the APP protein can lead to formation of amyloid plaques (Van Broeckhoven et al., 1990). As discussed previously, histopathological analysis of post-mortem AD brains showed Aβ to be the main constituent of amyloid plaques and subsequent genetic analysis identified *APP* gene mutations that were sufficient to cause AD (Wang et al., 1984, Masters et al., 1985, Selkoe et al., 1986, Bertram et al., 2010).

In the early 1990's a number of people attempted to bring the symptomatic, biochemical and genetic observations together and build a model for the sequence of events leading to the pathology of AD. The hypothesis that Aβ deposition is an early and causative event in the disease progression is central to the amyloid cascade model (Figure 1.4). Specifically, it was proposed that mis-regulation of APP processing and increased deposition of Aβ was the causative agent in AD pathology. Subsequent increases in neuritic plaques then caused, through unknown mechanisms, neuronal damage, activation of microglia and astrocytes, depletion of neurotransmitters and formation of NFTs. These downstream effects led to the clinical symptoms of dementia and gross neuronal cell loss (Hardy and Allsop, 1991, Selkoe, 1991a, Hardy and Higgins, 1992) (Figure 1.4). Due to significant sporadic AD cases, where no genetic linkage was apparent, it was proposed that environmental factors could also initiate Aβ deposition such as head trauma, as there was evidence of increased AD incidence following these events (Hardy and Higgins, 1992).

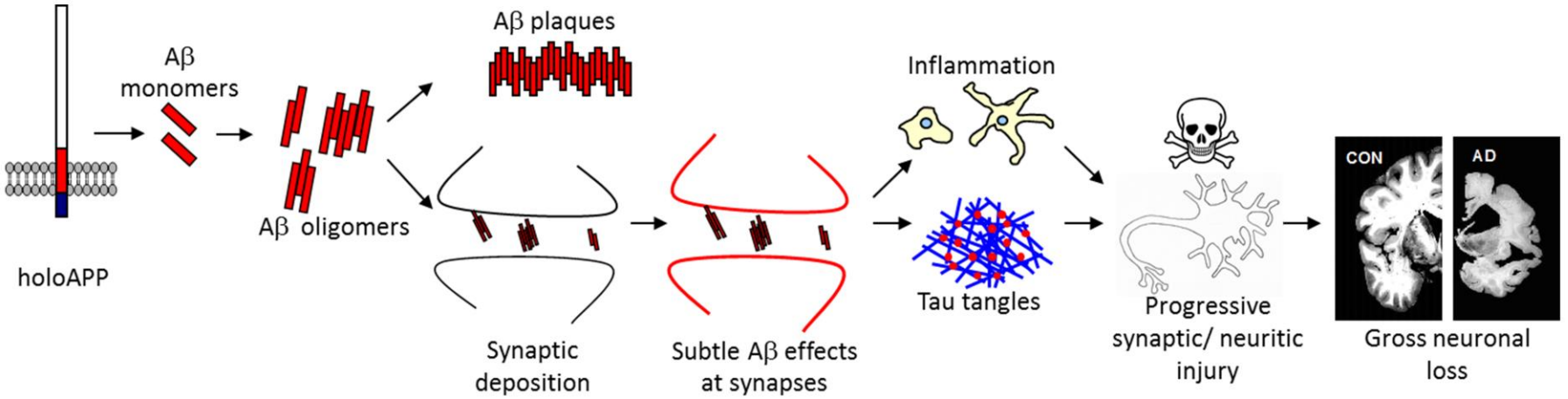



Figure 1.4: **Amyloid cascade hypothesis**

A schematic demonstrating the amyloid cascade where misregulation of APP, through missense mutations in APP or PSEN1/2 in FAD or through gradual increases in Aβ production with age in LOAD, leads to increased Aβ monomers which polymerise and generate toxic oligomers. These oligomers can then aggregate to form amyloid plaques or exert deleterious effects on synapses, subsequent hyperphosphorylation of tau and activation of inflammatory cells. This causes progressive synaptic and neuritic injury and eventual neuronal cell death and brain atrophy presenting as AD.

Direct neurotoxicity of Aβ is a contentious issue which has yet to be resolved. Aβ has convincingly been shown to have subtle effects at synapses (Walsh et al., 2002, Minano-Molina et al., 2011); however this has not been shown to translate into the neuronal loss observed in AD. Identification of tau mutations and creation of human tau transgenic mice showed that they do not lead to AD but to frontotemporal dementia and Parkinsonism, showing no amyloid deposition (Spillantini et al., 1998), suggesting mutations in tau are not the drivers of disease progression. A double tau (P301L)/APP (K670N/M671L) mutant however, increased the tangle pathology in the limbic system and olfactory cortex compared to the single tau transgenic, implying that APP can modulate tau pathology. (Lewis et al., 2001).

Aβ-mediated promotion of tau pathology and the tight correlation of NFT progression with cognitive decline, suggests that Aβ acts as an upstream activator of tau hyperphosphorylation, which then mediates Aβ-induced neurotoxicity. This lends further support to the amyloid cascade hypothesis that Aβ is the driver of pathogenicity in AD (Lewis et al., 2001, Rapoport et al., 2002, Roberson et al., 2007). Identification of further mutations in APP and realisation that they were clustered around the Aβ cleavage sites, and identification of mutations in the presenilins, strengthened the hypothesis that misregulation of Aβ production was central to AD progression (Hardy and Selkoe, 2002). Since its conception, the amyloid cascade hypothesis has shaped the progress of Alzheimer's research, and it remains the best model for the progression of AD.

## 1.5 Amyloid Precursor protein

APP is a highly conserved, type I transmembrane domain glycoprotein with orthologues identified from *Caenorhabditis elegans, Drosophila melanogaster, Xenopus laevis*, and *Danio rerio* (Zheng and Koo, 2011).

In Mammalia, APP is part of a family containing three homologous proteins: APP, APP like protein 1 (APLP1) and APLP2. These proteins show high sequence identity, with a large N-terminal extracellular domain, a single trans-membrane domain and

short C-terminal domain, although it is only APP that contains the Aβ sequence. APP and APLP2 undergo alternative splicing; in contrast only one isoform of APLP1 has been detected. All undergo complex glycosylation and processing to release soluble ectodomains (De Strooper and Annaert, 2000). APP, APLP1 and APLP2 are abundantly expressed in the brain, whilst APLP1 expression is entirely restricted to neurons, APP and APLP2 are also expressed in the periphery (Zheng and Koo, 2011), suggesting non-redundant roles for the family members.

The human APP gene encodes a 19 exon, 770 amino acid polypeptide (Yoshikai et al., 1991), which can undergo alternative splicing of exons 7, 8 and 15, all resulting in structural changes to the N-terminal, extracellular domain (De Strooper and Annaert, 2000). These alternative splicing events give rise to three APP isoforms in humans of 770, 751 and 695 amino acids; termed $APP_{770}$, $APP_{751}$ and $APP_{695}$ respectively. $APP_{695}$ is the primary source of APP in the brain, whilst its expression is barely detectable in other tissues (Tanaka et al., 1989). It is estimated that $APP_{695}$ mRNA accounts for 0.2% neuronal mRNA and shows little regional variability (Beyreuther et al., 1996), suggesting the physiological role of $APP_{695}$ in the brain is not region-specific. $APP_{770}$ and $APP_{751}$ are expressed at much lower levels in the brain, with the ratio of $APP_{770}$/$APP_{751}$/$APP_{695}$ estimated to be 1:10:20 (Tanaka et al., 1989).

## 1.5.1 APP structure

APP is a multi-domain protein, broadly segregated into the N-terminal, the trans-membrane and the C-terminal domains. Due to its large size and its properties as a transmembrane protein, the crystal structure of the holoprotein is yet to be solved. The N-terminal domain of APP contains a number of sub-domains: with the E1 and E2 domains showing high conservation across the APP family (Figure 1.5 (A)). The E1 domain consists of a heparin binding/growth factor like domain (GFLD) containing disulphide bridges essential for neurite outgrowth and mitogen activated protein kinase (MAPK) activation (Reinhard et al., 2005) and a metal (copper and zinc)

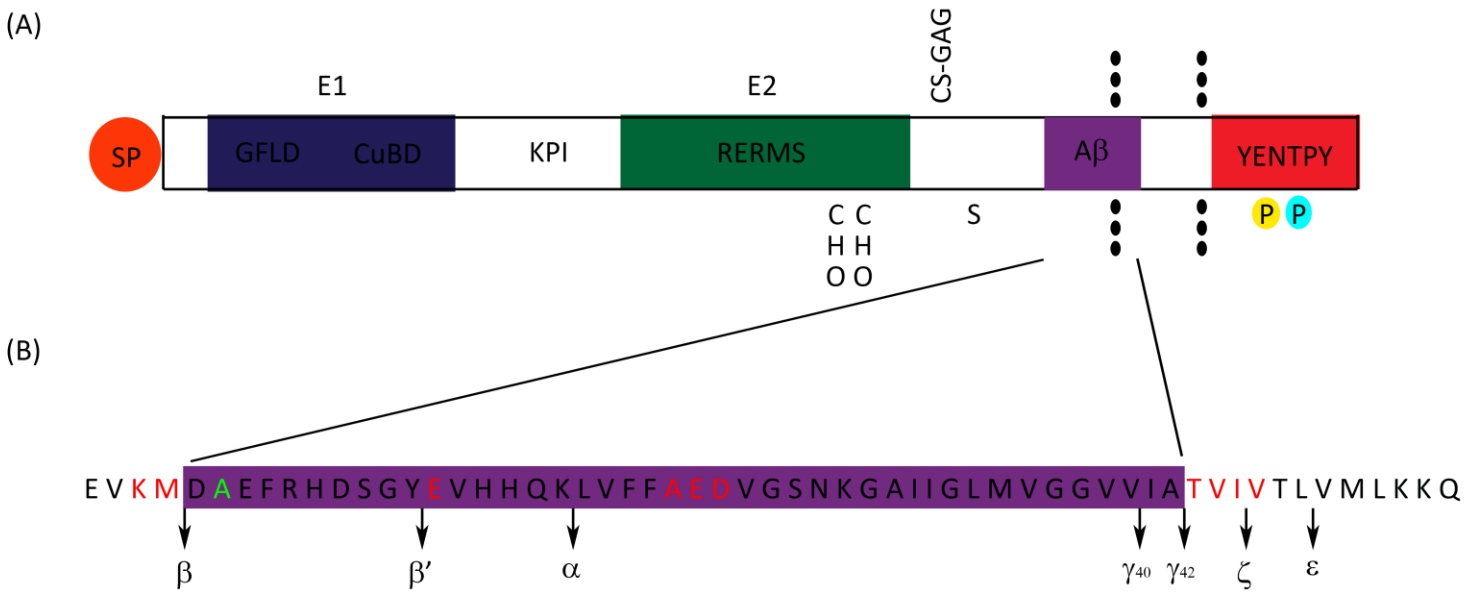



Figure 1.5: **APP structure and FAD linked mutations**

(A) The positions of the signal peptide (SP), growth factor-like domain (GFLD), copper (II) and zinc (II) binding domain (CuBD), Kunitz-type protease inhibitor domain (KPI), sequence implicated in growth promotion (RERMS), amyloid β (Aβ) and protein interaction motif (YENTPY). Above indicates the locations of the highly conserved E1 and E2 domains and the chrondroitin sulphate glycosaminoglycan attachment site (CS-GAG). Below indicates the locations of N- and O-glycosylation sites (C=O) and phosphorylation sites (yellow- ser/thr, blue- tyr). (B) Mutations in $APP_{695}$ (letters in red) occur in the Aβ sequence (purple box) or near the secretase cleavage sites (black arrows) and alter APP metabolism to favour Aβ production or aggregation. A single protective mutation (A673T) has been identified that reduces risk of AD (green) close to the β secretase cleavage site

binding domain (CuBD) that is responsible for the redox sensitivity of APP (Casella and Gullotti, 1993). The E1 domain is followed by a Kunitz type serine protease inhibitor domain (KPI) that is subject to alternative splicing and absent from $APP_{695}$ (Kitaguchi et al., 1988, Ponte et al., 1988). The E2 domain contains a RERMS sequence implicated in growth promotion (Ninomiya et al., 1993) and putative interaction sites for binding partners including a second heparin binding domain and two N-glycosylation sites (Zheng and Koo, 2011). This is followed by a chondroitin sulphate glycosaminoglycan attachment site (Shioi et al., 1995) which, along with the KPI domain is subject to alternative splicing to generate the three isoforms of APP. The transmembrane domain (TMD) contains the Aβ sequence, the first 28 residues are located outside the membrane and the last 12-14 are part of the trans-membrane sequence itself. The TMD also contains two putative sumoylation sites immediately prior to the Aβ sequence (Lys 587 and Lys 595), which have been associated with negative regulation of Aβ production (Zhang and Sarge, 2008). The TMD contains all known amino acid substitutions associated with FAD (Figure 1.5(B)). The C-terminal, intracellular domain contains the protein interaction motif YENTPY which is conserved from *C. elegans* to humans (Reinhard et al., 2005). The C-terminal domain also contains phosphorylation sites, including Thr668 ($APP_{695}$ numbering), the most well-characterised phosphorylation site, conserved in all homologs. Further sites include a second threonine site (Thr686) and two tyrosine phosphorylation sites (Tyr682 and Tyr687) (Jacobsen and Iverfeldt, 2009).

# 1.6 APP metabolism

Metabolism of APP is central to its function evidenced by rescue of the APP knockout phenotype by introduction of the APP metabolite, sAPPα. APP was one of the first proteins to be identified that undergoes a specific type of metabolism termed regulated intramembrane proteolysis (RIP) (Brown et al., 2000).RIP is an evolutionarily conserved pathway which mediates efficient release of membrane

anchored, bioactive molecules by proteolytic cleavage at a site within the membrane (Brown et al., 2000). RIP has been found in all organisms studied to date, controlling communication between cells and the extracellular environment (Lichtenthaler et al., 2011). Three families of intramembrane cleavage proteases (iCliPs) have been described: GxGD type aspartyl proteases, S2P metalloproteases and rhomboid serine proteases, each evolutionarily and structurally distinct.

RIP is tightly controlled, with deregulation associated with AD, Parkinson's disease (PD), and type 2 diabetes (Lemberg, 2011). Substrate tailoring, an initial cleavage event by a sheddase must occur to generate a membrane stub that is then accessible to the iCliPs, regulating substrate availability (Lemberg and Martoglio, 2002, Lichtenthaler et al., 2011). The initial shedding event of most RIP substrates is mediated by members of the ADAM family or aspartyl proteases BACE1 and BACE2 (Lichtenthaler et al., 2011). There is also intricate subcellular compartmentalisation of substrates and proteases, keeping the substrate and protease separate; only through compartment targeting is the process activated (Lemberg, 2011). RIP is associated with a range of disparate cellular functions; it can lead to relocation of a substrate domain, activation of a dormant activity or initiation of membrane protein degradation. A variety of membrane proteins including cytokines, growth factors, cell adhesion proteins, viral proteins and signal peptides are cleaved by RIP. Through these, the process is essential for a multitude of essential cellular functions including embryonic development, normal immune system function and the nervous system (Lemberg, 2011, Lichtenthaler et al., 2011).

In humans, APP is constitutively processed in all cell types by two RIP pathways, involving three proteases. The first pathway, termed the amyloidogenic pathway, gives rise to Aβ whilst the second pathway, termed the non-amyloidogenic pathway precludes Aβ formation. γ-secretase, the GxGD type aspartyl protease-containing complex responsible for the intramembrane proteolysis of APP, is common to both pathways. The pathways are differentiated by the sheddase enzymes responsible for the substrate tailoring of APP allowing the secondary cleavage to occur. This is the α-secretase activity for non-amyloidogenic and β-secretase activity for amyloidogenic processing (Haass et al., 2012) (Figure 1.6).

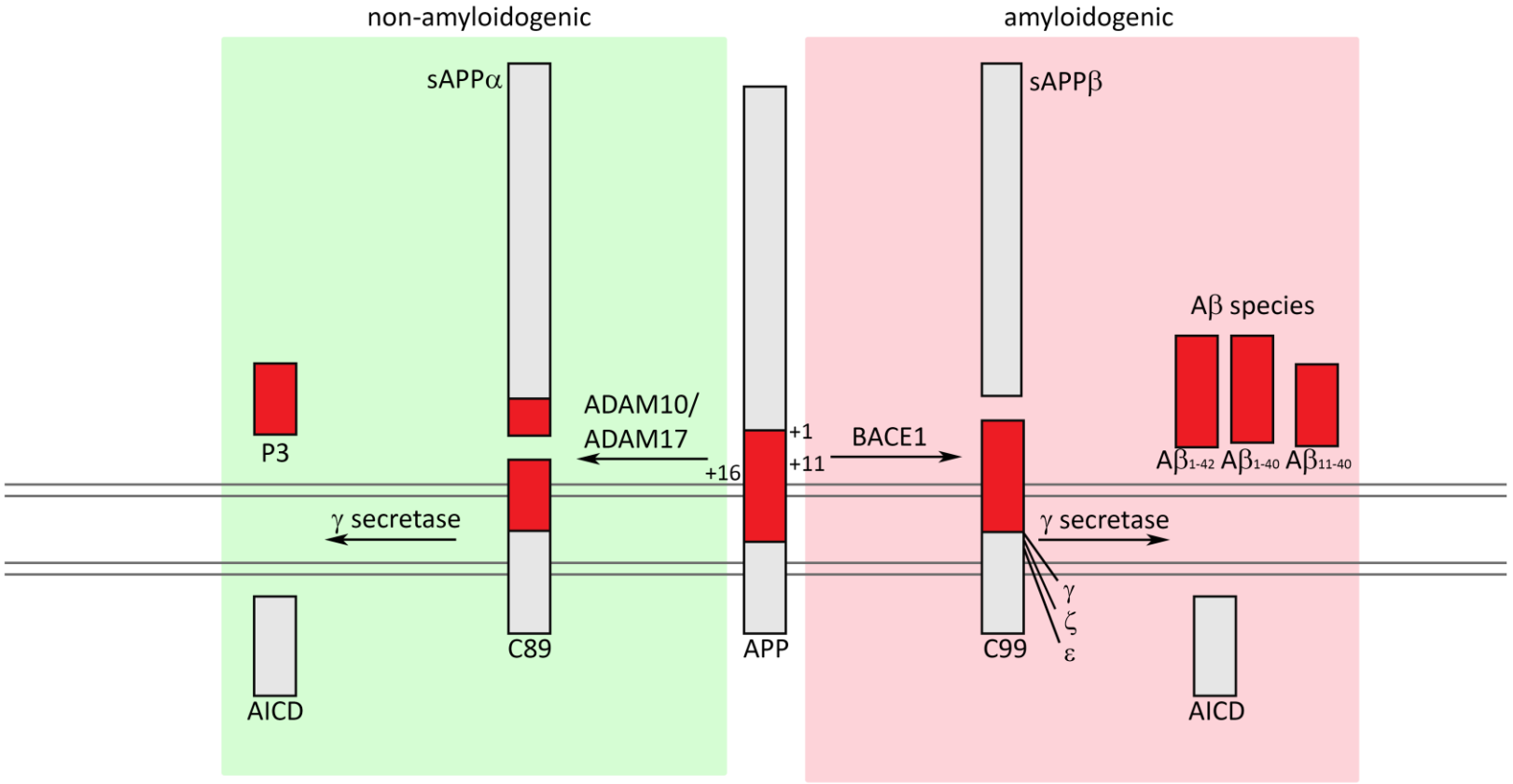

non-amyloidogenic
amyloidogenic
sAPPα
sAPPβ
Aβ species
P3
ADAM10/
ADAM17
+1
+11
+16
BACE1
Aβ1-42 Aβ1-40 Aβ11-40
γ secretase
γ secretase
γ
ζ
ε
C89
APP
C99
AICD
AICD

Figure 1.6: **APP processing pathways**

Primary cleavage of APP is performed by either α or β secretase activities following by intra-membrane proteolysis by γ secretase. Non-amyloidogenic processing (left) is initiated by α-secretase (ADAM10/17). Cleavage occurs between residues 16 and 17 of the Aβ sequence, precluding its formation, allowing release of soluble ectodomain sAPPα and retaining C89 (αCTF) in the membrane. Amyloidogenic processing (right) is thought to be the dominant pathway in neurons and is initiated by β secretase (BACE1). Cleavage occurs at 2 sites, the major site at the N-terminal of the Aβ sequence, however a second cleavage site at +11 (β') also exists, generating soluble ectodomain sAPPβ and retaining C99 (β'/β CTF) in the membrane. Following ectodomain shedding, the membrane tethered C89 and C99 undergo γ secretase cleavage, leading to release of P3 from C89 cleavage and Aβ release from C99 cleavage. Due to heterogeneity in γ secretase cleavage sites different amino acid length Aβ species are produced, the major isoforms being 40 and 42 amino acids in length ($A\beta_{40}$ and $A\beta_{42}$), N-terminally truncated Aβ species are generated by β' secretase activity. γ secretase cleavage of both pathways also releases the amyloid intracellular domain (AICD) into the cytoplasm.

Non-amyloidogenic processing is initiated by cleavage of APP between residues 16 and 17 of the Aβ sequence by the α secretase, precluding formation of Aβ and releasing the soluble ectodomain fragment, sAPPα (Sisodia, 1992b) (Figure 1.6: green box). This is followed by γ-secretase cleavage releasing the P3 peptide and the APP intracellular domain (AICD). The truncated Aβ sequence of the released P3 peptide does not appear to have toxic properties (Dulin et al., 2008).

In contrast, amyloidogenic processing is initiated by β-secretase cleavage at the N-terminal of the Aβ sequence, between Met670 and Asp671 (+1 site). This causes the release of a slightly shorter ectodomain fragment compared to α-secretase cleavage, termed sAPPβ (Figure 1.6: red box). This is followed by γ-secretase cleavage, releasing the AICD and due to its heterogeneous cleavage activity, a number of different length Aβ fragments. The major product is $A\beta_{40}$ and the minor product is $A\beta_{42}$ at an approximate ratio of 9:1 under physiological conditions (Lichtenthaler et al., 2011). Despite sequence specificity of the β-secretase enzyme, it can also cleave at a second site in Aβ termed the β' site (+11 site), the regulation of this preference has been associated with AD (Sisodia and St George-Hyslop, 2002).

The amyloidogenic and non-amyloidogenic pathways have been suggested to exist in equilibrium; the non-amyloidogenic pathway is the predominant APP cleavage route in peripheral cells whilst the amyloidogenic pathway is predominant in neurons due to increased β-secretase expression in comparison to non-neuronal cells (Colombo et al., 2012). Studies using genetic and pharmacological methods to increase α-secretase activity have shown a concomitant decrease in Aβ production and β-secretase activity (Postina et al., 2004, Bandyopadhyay et al., 2007). Overexpression of β-secretase was also shown to reduce α-secretase activity in the inverse relationship (Vassar et al., 1999, Kuhn et al., 2010). Observations from the study of the Swedish APP mutation which caused more efficient cleavage of APP by the β-secretase,, pointed to cellular compartmentalisation changes as a mechanism for altering the equilibrium (Haass et al., 1995b). However, study of endogenous secretase activities suggested the relationship was not bidirectional. β-secretase inhibition could not impact on the α-secretase pathway, although α-secretase

inhibition could increase β-secretase activity. This calls into question the physiological relevance of earlier overexpression observations and suggests a more complex relationship (Colombo et al., 2012).

# 1.7 Secretase enzymes

α-, β- and γ-secretase activities that cleave APP were described many years prior to identification of the protease responsible. Within the last few years, elucidation of α-secretase enzyme activity has completed the puzzle of secretase identities. These proteases are necessary and sufficient for the generation of the full range of metabolic products generated from RIP of APP.

## 1.7.1 Alpha secretase

α-secretase activity on APP was first reported over 20 years ago and was shown to cleave within the Aβ sequence between Lys16 and Leu17, precluding formation of the neurotoxic species (Esch et al., 1990, McDermott and Gibson, 1991). Further work characterised the enzyme activity showing it to be an integral membrane metalloendopeptidase of 105-120 kDa in size (McDermott and Gibson, 1991, Roberts et al., 1994).

### *1.7.1.1 Identity of α-secretase*

The A disintegrin and metalloprotease (ADAM) family of zinc metalloproteases were highly implicated as the protease responsible for α-secretase activity due to their identification as the sheddases of other RIP substrates such as notch and TNFα. Two ADAMs in particular, ADAM10 and ADAM17, were suggested as the most likely family members to cause cleavage of APP at the α- cleavage site (Black et al., 1997, Buxbaum et al., 1998, Artavanis-Tsakonas et al., 1999, Lammich et al., 1999, Slack et al., 2001). α-secretase activity of APP occurs constitutively and can also be stimulated by a variety of molecules. This stimulated response is termed 'regulated'

α-secretase activity. Through two studies, one in primary neurons and the other using conditional ADAM10 knockout mice, ADAM10 was shown to be responsible for the constitutive α-secretase activity (Jorissen et al., 2010, Kuhn et al., 2010). These were supported by the observation of coordinated expression of ADAM10 and APP in the brain, which was not seen with other implicated ADAMs (Karkkainen et al., 2000, Marcinkiewicz and Seidah, 2000). Furthermore transgenic studies showed ADAM10 overexpression in a mouse model of AD attenuated plaque pathology and enhanced sAPPα production. Conversely a mouse model expressing a dominant-negative mutant of ADAM10 displayed reduced sAPPα, increased amyloid plaques and showed learning deficiencies in the Morris water maze (MWM) (Postina et al., 2004, Schroeder et al., 2009).

The identity of the enzyme(s) responsible for the regulated α-secretase activity has not fully been elucidated, however ADAM10 itself can be upregulated in response to the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) (Kojro et al., 2006). In addition, ADAM17 expression is upregulated following phorbol myristate ester (PMA) treatment concurrent with changes in α-secretase activity and Aβ production. This was not seen in ADAM17 knockout cells, whilst constitutive α-secretase activity was maintained (Buxbaum et al., 1998). ADAM17 activity was also shown to be upregulated following treatment with M1 muscarinic agonists, this was in parallel with increases in sAPPα, which although not a direct link between ADAM17 and increased non-amyloidogenic processing, is indirect evidence for ADAM17 as the regulated α-secretase activity (Caccamo et al., 2009). Taken together these studies suggest a joint role for ADAM10 and ADAM17 as the regulated α-secretase activity.

### *1.7.1.2 Structures of ADAM10/17*

ADAMs are a family of approximately 750 amino acid, type I transmembrane, zinc proteinases characterised by their structure. There are 24 known *ADAM* genes in humans, however only half of these are active proteases (van Goor et al., 2009). They are generated as a zymogen with a signal peptide and prodomain at the N-

terminus. This is followed by a catalytic domain with a conserved zinc binding sequence (HEXGHXXGXXHD), a cysteine-rich disintegrin-like domain, a single transmembrane domain and a short cytoplasmic domain (Figure 1.7(A)). The prodomain holds the catalytic $Zn^{2+}$ ion and protease domain in an inactive state and in the case of ADAM10 it appears the prodomain also acts as a chaperone molecule during maturation (Endres and Fahrenholz, 2010). Evidence from the solved crystal structure of the human ADAM17 catalytic domain shows a central five-stranded β-sheet surrounded by five α-helices with a relatively flat active site (Maskos et al., 1998). ADAM10 and ADAM17 show little sequence specificity, cleavage is instead dependent on the presence of an alpha-helical conformation, substrate association with a membrane and the distance of a hydrolysable bond from the membrane (12-13 amino acids) (Sisodia, 1992a).

### *1.7.1.3 Alternative functions of ADAM10/17*

ADAM10 is responsible for cleavage of over 40 membrane proteins, making it one of the major brain sheddases. ADAM10 cleavage occurs at the plasma membrane and can occur on the same cell surface but also in trans, on a neighbouring cell surface (Lichtenthaler, 2011). ADAM10 is responsible for ectodomain shedding of proteins involved in a number of pathways, including the well characterised notch signalling pathway (Tian et al., 2008, De Strooper et al., 2010), E- and N-cadherin cleavage, involved in cell migration (Maretzky et al., 2005, Reiss et al., 2005) and the cleavage of low affinity immunoglobulin E receptor in the immune system (Weskamp et al., 2006). The ADAM10 knockout mouse showed embryonic lethality at E9.5 displaying severe developmental defects in the CNS and heart; this phenotype closely resembles a loss of notch signalling phenotype, one of the major substrates of ADAM10 (Hartmann et al., 2002). In the CNS, the classic cadherins are involved in axon guidance and synaptogenesis during development, and synaptic plasticity in adults. Cleavage by ADAM10 leads to disassembly of adherens junctions at the synapse and modification of synaptic strength (Maretzky et al., 2005, Malinverno et al., 2010).

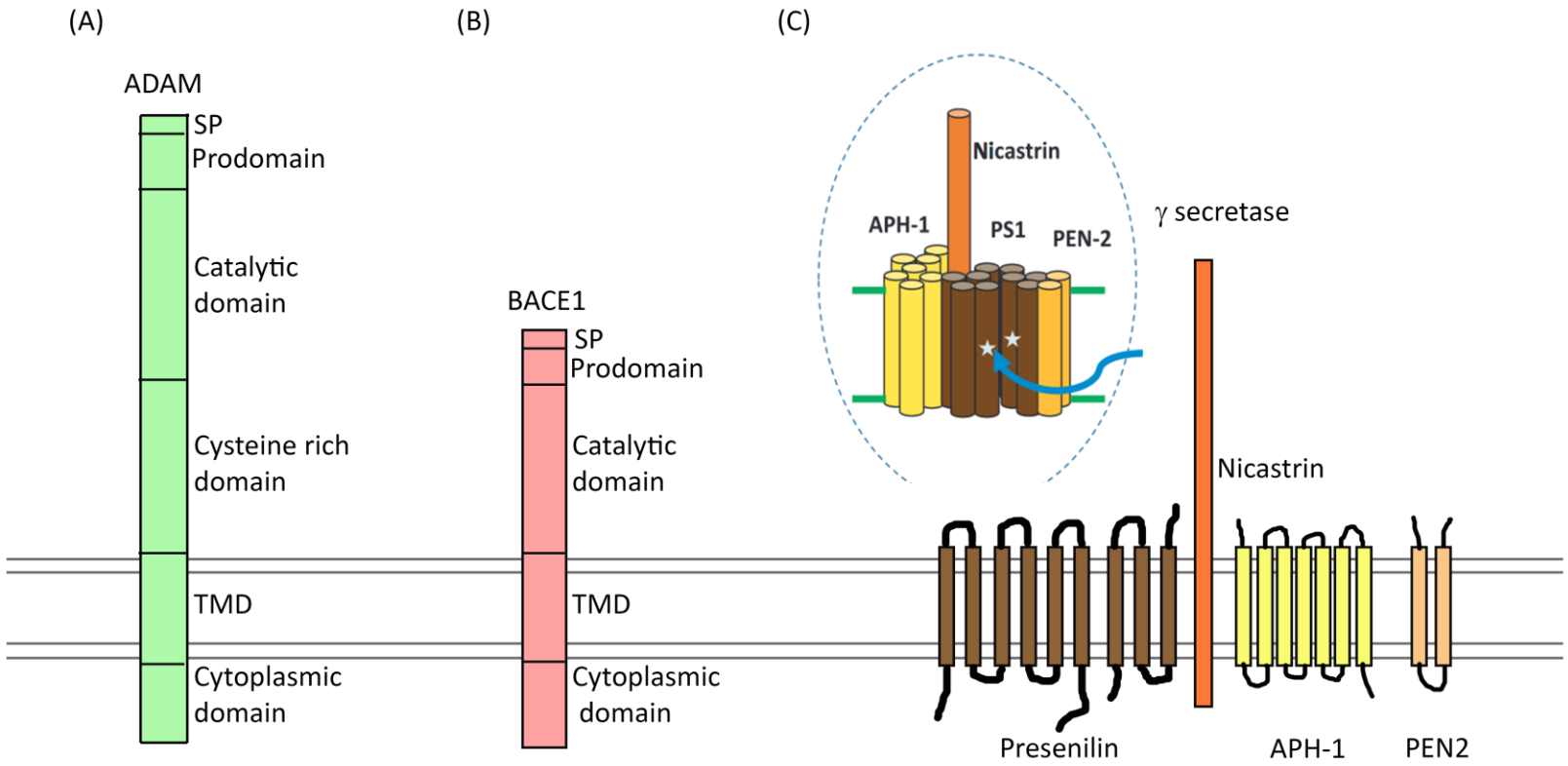

(A)
ADAM
SP
Prodomain
Catalytic
domain
Cysteine rich
domain
TMD
Cytoplasmic
domain
(B)
BACE1
SP
Prodomain
Catalytic
domain
TMD
Cytoplasmic
domain
(C)
Nicastrin
APH-1
PS1
PEN-2
γ secretase
Nicastrin
Presenilin
APH-1
PEN2

Figure 1.7: **Structure of secretase enzymes**

(A) α-secretase activity is mediated by two A disintegrin and metalloprotease (ADAM) family members, ADAM10 and ADAM17, which have very similar structures. The positions of the signal peptide (SP), prodomain, which keeps the enzyme in an inactive state, catalytic domain with conserved zinc binding sequence, a cysteine-rich disintegrin-like domain, a single transmembrane domain (TMD) and cytoplasmic domain are indicated. (B) The β-secretase enzyme, BACE1, is a single transmembrane aspartyl protease. The positions of the signal peptide (SP), prodomain which mediated correct folding of BACE1, catalytic domain which contains two conserved motifs, GTGS and DSGT containing the catalytic aspartates, the single transmembrane domain (TMD) and cytoplasmic domain which contains trafficking and recycling motifs. (C) γ secretase is composed of four essential proteins: presenilin (PS), nicastrin, anterior pharynx-defective 1 (APH1) and presenilin enhancer 2 (PEN2) in a 1:1:1:1 ratio. Presenilin undergoes endoproteolytic cleavage between transmembrane domains (TMD) 6 and 7. The subunits form a barrel-like structure with a central hydrophilic chamber (blue arrow) where APP is cleaved by a di-aspartate active site (stars) (Image taken from (St George Hyslop and Fraser, 2011)).

Like ADAM10, ADAM17 has a broad range of substrates and cleavage occurs at the plasma membrane (Vingtdeux and Marambaud, 2012). It is critical for the activation of TNFα and endothelial growth factor (EGF) receptor ligands, regulators of inflammation and cell growth (Black et al., 1997, Sahin et al., 2004). *ADAM17* gene depletion caused perinatal lethality displaying severe heart development defects whilst the knockout mouse phenotype closely resembles TNFα knockout mouse phenotype, showing embryonic lethality (Saftig and Reiss, 2011).

#### *1.7.1.4 Regulation of α-secretase activity*

α-secretase ectodomain shedding can be modulated at multiple levels including transcription, phosphorylation, protein maturation and trafficking. ADAMs are regulated by tissue inhibitors of metalloproteases (TIMPs); ADAM10 is inhibited by TIMP1 and TIMP3 whilst ADAM17 is inhibited by TIMP3 (Vingtdeux and Marambaud, 2012). Cholesterol has been implicated in regulation of these interactions through cholesterol mediated activation of liver X receptors. These down-regulate TIMP3 leading to increased ADAM10 and ADAM17 activity (Amour et al., 2000, Hoe et al., 2007). ADAM10 activity has also been shown to be negatively regulated by reversion-inducing cysteine-rich protein with Kazal motifs (RECK), which is crucial for appropriate ADAM10-mediated notch signalling during development (Muraguchi et al., 2007). Up-regulation of ADAM activity also occurs through direct activators of protein kinase C (PKC) which phosphorylate the ADAM protein and targets it to the plasma membrane. Insulin-like growth factor 1 (IGF1)-mediated α-secretase activity stimulation through PI3-K- and cdk5-mediated signalling pathways has also been reported (Adlerz et al., 2007).

Synaptic activity can further regulate α-secretase activity, through interaction of ADAM10 with synapse associated protein 97 (SAP97), calcium ($Ca^{2+}$)-dependent NMDAR activation affects its distribution, targeting ADAM10 to the post-synaptic membrane, leading to increased α-secretase activity (Marcello et al., 2007).

## 1.7.2 Beta secretase

### *1.7.2.1 Identity of β- secretase*

The protease responsible for the β-secretase cleavage of APP was the first secretase to be identified. It was isolated and characterised by five separate groups in 1999. Together, these studies showed it to be a 501 amino acid, transmembrane aspartic protease that has been termed beta-site APP-cleaving enzyme 1 (BACE1) (Hussain et al., 1999, Sinha et al., 1999, Vassar et al., 1999, Yan et al., 1999, Lin et al., 2000). BACE1 has a low optimum pH and is predominantly localised to early endosomes and the TGN. Its highest tissue expression is in the pancreas and brain, more specifically neurons (Vassar et al., 2009). BACE1 is only found in vertebrates, with no homologs in *C. elegans* or *Drosophila*, suggesting a role in vertebrate specific systems such as the complex immune system and the brain. BACE1-/- mice are viable and fertile (Luo et al., 2001), however they present with a higher risk of premature mortality, impaired memory, reduced axonal myelination and altered synaptic plasticity. There is no cerebral Aβ produced indicating BACE1 is the major if not only β-secretase in the brain (Vassar et al., 2009). When BACE1-/- mice are crossed with APP transgenic mice, Aβ generation, amyloid pathology and cognitive deficits are abrogated, further supporting BACE1 as the major β-secretase enzyme (Harrison et al., 2003, Ohno et al., 2004, Laird et al., 2005, Hu et al., 2010).

### *1.7.2.2 Structure of BACE1*

In humans, there are four different BACE1 isoforms due to alternative splicing of exons 3 and 4, of 501, 476, 457 and 432 amino acids in length. These show different β-secretase activities and are thought to be responsible for the different activities of BACE1 in the brain and pancreas (Mowrer and Wolfe, 2008). Soon after identification of BACE1, a second homologous protein termed BACE2 was discovered, this however has low neuronal expression and does not have the same cleavage activity on APP as that seen with the β-secretase.

BACE1 is synthesized in the endoplasmic reticulum (ER) as a zymogen with a signal peptide and short prodomain. The prodomain does not inhibit BACE1 activity, but mediates correct folding of BACE1 in the late Golgi network and then is removed by furin in the TGN (Bennett et al., 2000). C-terminal to the prodomain is the catalytic domain, containing two conserved motifs, DTGS and DSGT, with catalytic aspartate residues; mutation of either of these annihilates enzyme activity (Hussain et al., 1999, Bennett et al., 2000, Hong et al., 2000). This is followed by the TMD and then the cytoplasmic domain, which contains motifs that dictate trafficking and recycling of BACE1 (Figure 1.7(B)). The crystal structure of the catalytic domain was solved in 2000, revealing N and C-terminal lobes with the substrate binding and catalytic active sites in the cleft (Hong et al., 2000). Unlike the ADAMs, which show little sequence specificity, the active site of BACE1 accommodates 12 residues at positions P4' to P8 with residue preferences at each site. P1 is the most stringent, getting more relaxed towards the outer sites (Wang et al., 2013).

### *1.7.2.3 Alternative functions of BACE1*

It was thought that the $\alpha$ secretases ADAM10 and ADAM17 were the major sheddases in the brain, whilst BACE1 was responsible for shedding of a small number of proteins. Those originally identified included APP, neuregulins and $\beta$2 subunits of voltage gated sodium channels (Hu et al., 2006, Kim et al., 2007, Hu et al., 2008, Gersbacher et al., 2010). This was recently challenged following identification of 34 neuronal membrane proteins whose ectodomain shedding was affected by BACE1 inhibition (Kuhn et al., 2010). Of these several were almost exclusively BACE1 substrates such as seizure protein 6 like 1 (SEZ6L1) and the APP homolog APLP1, whilst others, such as APP, showed significant levels of BACE1 dependence. These findings suggest BACE1 is also a major sheddase in the brain, responsible for cleavage in a wide range of developmental cellular pathways such as neurite outgrowth, myelination, synapse formation and axon guidance (Kuhn et al., 2010).

#### *1.7.2.4 Regulation of BACE1*

BACE1 expression is upregulated in response to energy deprivation and elevated levels of stress-related transcription factors such as nuclear factor kappa-light-chain-enhancers of activated B cells (NFκB) and hypoxia inducible factor 1 (HIF1) (Sambamurti et al., 2004, Velliquette et al., 2005, Zhang et al., 2007). Changes in the lipid environment also stimulate BACE1 activity *in vitro* (Kalvodova et al., 2005). BACE1 activity is further regulated by its subcellular localization through protein interactions predominantly via its cytoplasmic domain. Interaction with the myelin component reticulon retains BACE1 in the ER, spatially restricting its activity (He et al., 2007). Golgi-localised γ-ear-containing ARF-binding proteins (GGAs) bind to the cytoplasmic domain of BACE1 and regulate its trafficking from the late Golgi to endosomes. Decreased levels of these proteins lead to enhanced localization of BACE1 in its favoured endosomal compartment and also reduced trafficking to lysosomes where BACE1 is degraded, thus increasing BACE1 activity (He et al., 2005).

### 1.7.3 Gamma secretase

In 1993, 'γ-secretase' was the name given to the unusual proteolytic activity that released Aβ within the TMD of APP. The same paper predicted that it would be the same protease responsible for the C-terminal cleavage of the non-amyloidogenic pathway (Haass and Selkoe, 1993).

#### *1.7.3.1 Identity of γ-secretase*

Two genes were identified on chromosome 1 and chromosome 14 that segregated with identified cases of FAD. These genes were confirmed as coding for the catalytic activity of γ-secretase and were called *presenilin 1* and *presenilin 2*. Through generation of presenilin knockout primary cultures, the presenilins were shown to be critical for Aβ generation (De Strooper et al., 1998, Herreman et al., 1999), thus

identifying them as the catalytic components of γ-secretase. Presenilin 1 was shown to be responsible for the majority of γ-secretase activity. Following identification, biochemical characterisation of the presenilins highlighted they were not solely responsible for the γ-secretase activity through a number of observations:

1. cellular presenilin levels were tightly controlled by the levels of another component (Thinakaran et al., 1997),
2. when presenilins were co-fractionated with the γ-secretase activity they were part of a very high molecular weight complex (Li et al., 2000),
3. mutagenesis of the key catalytic aspartate residues not only ablated activity but also reduced the size of the large complex (Yu et al., 2000a).

Through interactions studies and genetic screens nicastrin, anterior pharynx defective 1 (APH1) and presenilin enhancer 2 (PEN2) were identified as the other components of the complex and together the four proteins were shown to be necessary and sufficient for γ-secretase activity in a 1:1:1:1 stoichiometry (Yu et al., 2000b, Francis et al., 2002, Takasugi et al., 2003) (Figure 1.7 (C)).

### *1.7.3.2 Structure of γ-secretase*

All proteolytic enzymes require an activated water molecule to execute their catalysis. γ-secretase is one of a growing number of enzymes identified that is able to execute catalysis in the hydrophobic environment of the lipid bilayer of a membrane (Lichtenthaler et al., 2011). Due to this unusual capability, the structure of the γ-secretase is of significant interest. Difficulties due to its size and membrane association means the crystal structure of the γ-secretase complex has not been solved, however low resolution structural studies predict it to be a barrel-like structure with a central aqueous cavity (Lazarov et al., 2006, St George-Hyslop and Fraser, 2012).

Presenilins have two catalytic aspartate residues in TMD 6 and 7, closely opposed to each other. Cysteine scanning mutagenesis of these two TMDs identified a number of residues that were water accessible including both catalytic aspartates,

biochemically supporting the presence of a hydrophilic channel in the membrane (Tolia et al., 2006). Due to the presence of this cylindrical interior chamber the proteolytic site is occluded from the hydrophobic environment, allowing access for crucial water molecules (De Strooper et al., 2012).

The presenilins are synthesised in the ER as 50 kDa precursor proteins with 9 transmembrane domains with a cytoplasmic N-terminal and luminal/extracellular C-terminal. Cleavage between TMD 6 and 7 at the final stage of maturation produces an N-terminal 30 kDa and C-terminal 20 kDa fragments which remain stably associated (Thinakaran et al., 1996, Fukumori et al., 2010).

Nicastrin is a type 1 transmembrane glycoprotein with a large extracellular domain which initially interacts with APH1 to create a scaffold in the ER for presenilin to associate with, it is also thought to be the 'gate-keeper', restricting substrate access to the active site in the mature complex (De Strooper et al., 2010, St George-Hyslop and Fraser, 2012).

APH1 is an integral membrane protein that exists as two homologs in humans, APH1a and APH1b of which APH1a can also undergo alternative splicing (Gu et al., 2003). It contains 7 TMDs and is the most stable part of the complex. Due to its different isoforms it may alter the catalytic activity of γ-secretase, some favouring formation of Aβ.

PEN2 contains two TMDs and is the final component to associate with the complex. This stabilises the complex via a conformational change, preventing its degradation by the proteasome and allowing endoproteolysis of presenilin, causing full activation of the complex (De Strooper et al., 2010). The full complex is a highly hydrophobic, 19 TMD protease localised to the plasma membrane and endosomes (Figure 1.7(C)).

### *1.7.3.3 Alternative functions of γ-secretase*

γ-secretase cleaves type 1 transmembrane proteins with short ectodomains with little sequence specificity, although more than 50 residues N-terminal to the

membrane inhibits activity. Over 90 substrates of γ-secretase have been identified, implicating γ-secretase in regulation of critical signalling pathways in development, immune responses and cellular differentiation (Wakabayashi and De Strooper, 2008, Jurisch-Yaksi et al., 2013). Single and double knockout *PSEN* mice die perinatally and during early embryonic development respectively (Shen et al., 1997, Donoviel et al., 1999, Herreman et al., 1999). They display disorders in neuronal migration, midline defects in the body wall, defective somatogenesis and show haemorrhages into the brain parenchyma (Shen et al., 1997, Wong et al., 1997, De Strooper et al., 1998, Hartmann et al., 1999). Mutations in *PSEN* are associated with five diseases: AD, fronto-temporal dementia, acne inversa, dilated cardiomyopathy and breast cancer, underlining the critical role of γ-secretase across a wide range of body systems including the heart, brain, vasculature and immune system (Li et al., 2006, To et al., 2006, Wang et al., 2010, Cruts et al., 2012).

Along with APP, notch is the most famous γ-secretase substrate and is thought to be responsible for the major phenotypes of *PSEN* knockout mice. During development, γ-secretase-mediated notch signalling regulates cell proliferation, survival, positioning and differentiation through a signalling mechanism involving ligand-dependent release of the notch intracellular domain (NICD). The NICD then translocates to the nucleus and modulates gene transcription (Kopan and Ilagan, 2009). In adulthood, notch signalling still has critical functions including modulation of stem cell maintenance and cell fate decisions (Grabher et al., 2006, Lavado and Oliver, 2014).

Many substrates of γ-secretase have brain-specific functions: for example, ephrin B and the ephrin B receptor and netrin and its receptor, deleted in colorectal cancer (DCC) which are involved in axonal guidance (Bai et al., 2011, Georgakopoulos et al., 2011). γ-secretase plays an important role at the synapse in maintaining proper neuronal communication. The cell adhesion protein neuroligin, present at the post-synaptic membrane, binds its pre-synaptic ligand neurexin and initiates γ-secretase dependent signalling across the synapse. Furthermore, γ-secretase-dependent ephrinA4 receptor activation regulates dendritic spine morphogenesis through activation of the Rac signalling pathway (Inoue et al., 2009, Suzuki et al., 2012).

Down-regulation of γ-secretase expression leads to an autoimmune phenotype, predominantly through a notch dependent impairment of T cell receptor signalling (Grabher et al., 2006). Other γ-secretase substrates are also associated with the immune system, although the functional relevance of their γ-secretase cleavage is not clear. These include CD46, lipoprotein receptor related protein 1 (LRP1) and a number of interleukin receptors (Jurisch-Yaksi et al., 2013).

γ-secretase is also responsible for cleavage of growth factors including vascular endothelial growth factor (VEGF) and EGF through which γ-secretase plays a crucial role in angiogenesis and cellular migration and proliferation (Ni et al., 2001, Cai et al., 2006).

### *1.7.3.4 Regulation of γ-secretase*

Several different cleavage events are mediated by the γ-secretase complex (Figure 1.6): the ε cleavage generates soluble intracellular domain fragments such AICD (APP). The ζ cleavage generates small, short lived fragments and the γ cleavage between residues 38 and 43 gives rise to the neurotoxic amino terminal Aβ fragments and P3 fragment from the non-amyloidogenic pathway (St George-Hyslop and Schmitt-Ulms, 2010). It has suggested that these cleavages occur sequentially from ε to γ and that different PSEN and APH subunit combinations affect the preferred activity of the complex. PSEN2 containing γ-secretases are suggested to be less effective producers of Aβ and APH1b-containing γ-secretases favour production of longer Aβ peptides (Acx et al., 2013).

Non-essential protein components affect γ-secretase activity on specific substrates. Down-regulation of transmembrane emp24 domain-containing protein 21 (TMP21), caused an increase in γ-secretase mediated Aβ generation with no effect on the ε activity responsible for notch cleavage, and had no effect on AICD mediated transcriptional regulation of proteins such as neprilysin (Chen et al., 2006, Dolcini et al., 2008). More recently another protein, the gamma secretase activating protein (GSAP) was identified that via direct interaction with the APP, is able to selectively

affect the γ cleavage activity whilst the ε cleavage activity remains unaffected (He et al., 2010). These findings are finally starting to help elucidate how γ-secretase specificity is conferred, although there are still significant gaps in our understanding.

# 1.8 Regulation of APP processing

## 1.8.1 Spatial regulation

As already discussed, APP undergoes a particular type of proteolytic cleavage termed RIP. The spatial and temporal localisation of the substrates and proteases play a critical role in the regulation of this type of processing.

APP is synthesised and N-glycosylated in the ER. Whilst in this state it is not subject to cleavage by proteases. Following trafficking to the Golgi it is O-glycosylated, which allows release into the TGN and the late secretory pathway (Suzuki and Nakaya, 2008) (Figure 1.8(1)). Following delivery to the cell surface, APP co-localises with the non-amyloidogenic sheddases, the α secretase enzymes, ADAM10/17 (Figure 1.8(2)). The localisation of α-secretase at the cell surface was deduced from endocytosis inhibition experiments. These showed increased cell surface APP concurrent with increased sAPPα and decreased Aβ (Koo and Squazzo, 1994). Further studies also showed ADAM10 and ADAM17 interact via an SH3 domain in their cytoplasmic tails with SAP97, ensuring localisation at the synaptic membrane (Peiretti et al., 2003, Marcello et al., 2007).

At the cell surface, APP has a relatively rapid endocytosis rate, internalised via the clathrin-dependent pathway (Figure 1.8(3)). Internalisation into the endosomal-lysosomal pathway presents APP to the β secretase (BACE1) activity, concentrated in these compartments (Small and Gandy, 2006) (Figure 1.8(4)). Evidence for β-secretase localisation includes the presence of a sorting signal (DXXLL) in the cytoplasmic tail of BACE1, mediating trafficking between the TGN to endosomes (He et al., 2005) (Figure 1.8(5)) and FRET analysis of the BACE1: APP interaction that

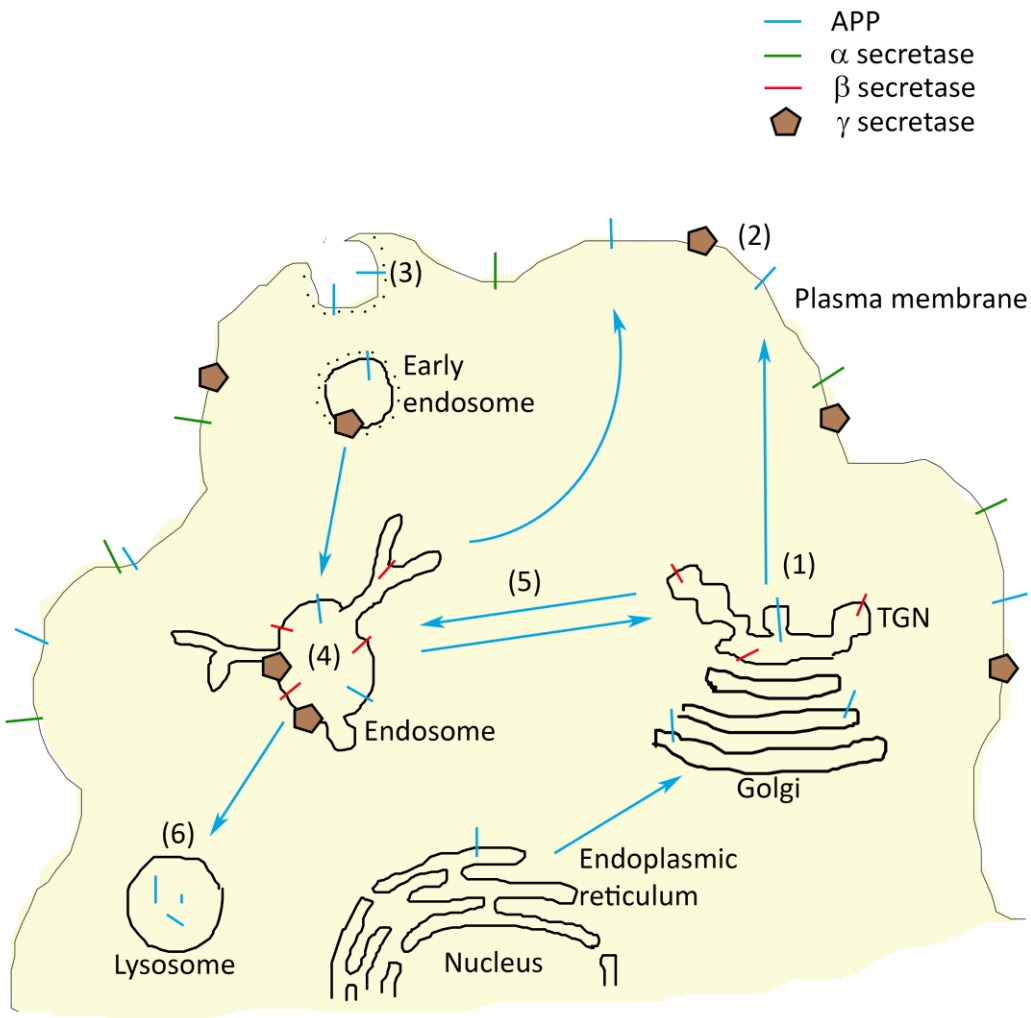



Figure 1.8: **Trafficking and proteolysis of APP**

APP (blue bars) matures through the constitutive secretory pathway (1). At the cell surface it is susceptible to α-secretase (green bars) and γ secretase (brown pentagons) activities. Once at the cell surface APP (2) is rapidly endocytosed and trafficked through the endocytic pathways (3) where it is susceptible to β secretase (4) (red bars) and γ secretase activities or recycled back to the plasma membrane. As well as in the endosomes, β secretase activity is also present in the trans-golgi network (TGN) and trafficking of APP regulates the availability of substrate for amyloidogenic processing in these compartments (5). A small fraction of APP is degraded in lysosomes (6).

showed greatest efficiency in the endosome (Kinoshita et al., 2003). Furthermore, endocytosis blockade experiments reduced Aβ production (Small and Gandy, 2006) and immunofluorescence studies showed BACE1 staining co-localised with the endosomal marker, transferrin receptor (Huse et al., 2000). BACE1 is also present on the cell surface, however due to spatial segregation, possibly due to membrane lipid content and the low optimal pH for BACE1 activity, this is not a major APP cleavage site (Marquer et al., 2011).

The localisation of the γ-secretase cleavage event has been difficult to elucidate due to its commonality to both pathways and its subunit composition. Components of the γ-secretase have been identified in most cellular compartments including the cell surface, golgi and endosomes; however this does not necessarily reflect mature, active γ-secretase complexes. Indirect measures of the γ-secretase complex utilising fluorescent reporters or Aβ production as surrogate markers of γ-secretase activity, have shown it to be focussed at the plasma membrane and in endosomes (Caporaso et al., 1994, Kaether et al., 2006). This corresponds with the localisation of α- and β-secretase activities and suggests γ-secretase is not the rate limiting enzyme in Aβ generation, but that it is presentation of APP to the endosomal BACE1 sheddase.

## 1.9 APP protein interactions

Discrete subcellular localisations of secretase enzyme activities means the regulation of APP processing can be finely controlled by APP trafficking and competitive interactions with other proteins. Interactions with binding proteins at its cytoplasmic domain can alter the stability of APP at the cell surface and the likelihood of APP internalisation. LRP1 interacts with APP at the plasma membrane, increasing its rate of endocytosis (Marzolo and Bu, 2009). In contrast, apolipoprotein E receptor 2 (ApoER2), which is highly expressed in the brain, stabilises APP at the cell surface through interactions via f-spondin (N-terminal domain) and Fe65 (cytoplasmic domain). Due to its lower endocytosis efficiency, ApoER2 slows the APP endocytosis rate whilst bound (Willnow and Andersen,

2013). Through the study of genes down-regulated in sporadic AD, sorting-related receptor with A-type repeats (SORLA) was shown to regulate APP (Scherzer et al., 2004). SORLA directly interacts with APP and stabilises the holoprotein, preventing both amyloidogenic and non-amyloidogenic processing pathways and also sequesters APP from the endosomes to the TGN through retromer transport, spatially preventing secretase cleavage (Willnow and Andersen, 2013).

## 1.9.1 APP adaptor protein interactions

APP protein interactions are often mediated by a group of cytoplasmic adaptor proteins, the most common being Mint/X11s, Fe65 and JIPs (Suzuki and Nakaya, 2008).

### *1.9.1.1 X11 family*

The X11 family of multi-domain adaptor proteins have an evolutionarily highly conserved interaction with APP. In humans, they are a three protein family: X11$\alpha$, X11$\beta$ and X11$\gamma$. X11$\alpha$ and $\beta$ are expressed primarily in neurons, predominantly in the TGN and at the plasma membrane. Binding of either X11$\alpha$ or X11$\beta$ to APP stabilises the holoprotein and increases its half-life, reducing the levels of A$\beta$ production (Miller et al., 2006, Rogelj et al., 2006). The X11 family are also known as Munc interacting proteins (MINTs) due to their interaction with munc18, a synaptic vesicle docking protein. This interaction has been shown to synergistically enhance the stabilising effect of X11 on APP, further reducing A$\beta$ production (Ho et al., 2002). X11s can also tether cargo to molecular motors. X11$\alpha$, as part of a larger protein complex, binds the dendritic motor kinesin superfamily motor protein 17 (KIF17) whilst an X11$\beta$/alcadein complex was co-purified with the kinesin heavy chain (Setou et al., 2000, Araki et al., 2003) These interactions suggest the X11 family play a role in axonal and dendritic transport, although direct evidence is lacking, therefore they may modulate APP processing through regulation of APP trafficking.

### *1.9.1.2 Fe65 family*

In humans, Fe65, Fe65L1 and Fe65L2 are another three protein family of multidomain adaptor proteins, however only Fe65 is brain enriched. They all contain an N-terminal WW domain and 2 C-terminal phosphotyrosine binding (PTB) domains (McLoughlin and Miller, 2008). As well as mediating interactions such as that between LRP or ApoER2 and APP as described previously, Fe65 also stabilises the γ-secretase-mediated APP cleavage product AICD via its YENTPY motif (Fiore et al., 1995, McLoughlin and Miller, 1996). Phosphorylation at Thr668 of APP modulates this interaction, with phosphorylation causing a conformational change, which enables Fe65 binding (Chang et al., 2006). Binding of the AICD to Fe65 targets it to the nucleus where it has been reported that a further adaptor, the histone acetyltransferase Tat interacting protein 60 (Tip60), associates to form a transcriptionally active complex (Cao and Sudhof, 2001, Kimberly et al., 2001). It remains controversial whether it is AICD or Fe65 that is the dominant transcriptional activity (Perkinton et al., 2004, Yang, 2006), however studies have implicated the complex in the transcriptional regulation of GSK3β, BACE1, neprilysin and APP (Miller et al., 2006) suggestive of feedback loops in APP regulation. Due to competition for the same YENTPY motif on APP, X11 proteins inhibit Fe65 interactions with APP, therefore indirectly inhibiting the transcriptional activity of APP (Rogelj et al., 2006).

### *1.9.1.3 JIP*

The scaffold proteins, C-Jun N-terminal kinase (JNK) interacting proteins (JIPs), can also interact with the YENTPY motif of APP (King and Scott Turner, 2004). JIP1a and JIP2 bind weakly to APP and do not affect its processing. In contrast, JIP1b causes evolutionarily conserved enhancement of JNK mediated, neuron specific phosphorylation at the critical Thr668 residue, the major regulatory phosphorylation site of APP. JIP1b also interacts with kinesin light chain-1,

suggesting a similar trafficking role to the X11 family (Taru et al., 2002, Inomata et al., 2003).

Although much is left to be elucidated about the regulation of APP and how its trafficking affects this, the picture is becoming clearer as to the complexity surrounding the pathway through which APP will be processed and the level to which it will be processed. The physiological spatial segregation of the secretase enzymes and tight control of trafficking of APP highlights the importance of studying modulators of APP processing in primary neurons, where all neuronal specific cellular compartments are present and functional.

# 1.10 Degradation of APP fragments

Full length $APP_{695}$ has a half-life of 10 minutes at the plasma membrane where its physiological role is thought to take place and its half-life in the cell is approximately an hour (Gralle and Ferreira, 2007). Although proteolytic fragments of APP are known to have independent functions and to be more stable that the full length protein, little specific information is available about their degradation pathways. The exception to this is degradation and clearance of Aβ.

Aβ production in the brain is much higher than in the periphery. Excess accumulation is prevented by drainage from the extracellular space into the muscle walls of arterioles (Weller et al., 2000, Bell et al., 2009). Specific enzymes have been identified that are involved in the degradation of Aβ including neprilysin, insulin degrading enzyme (IDE), endothelin converting enzymes 1 and 2 (ECE1 and ECE2), plasmin, matrix metalloproteinases (MMP2 and MMP9) and cathepsin B (De Strooper, 2010). These have distinct subcellular localisations, suggesting complimentary activities in ensuring efficient Aβ degradation (Turner and Nalivaeva, 2006). Supporting this, genetic reduction in expression of neprilysin and ECE1 both lead to an increase in Aβ accumulation and polymorphisms in ACE are associated with LOAD risk (Eckman et al., 2001, Hu et al., 2001, Iwata et al., 2001).

Also a double knock-out of neprilysin and ECE1 further enhanced Aβ accumulation, suggesting the proteases are complimentary (De Strooper, 2010).

# 1.11 Functions of APP

Despite the intense research activity focussed on APP, its physiological function is yet to be fully elucidated, although its structure and processing give clues to potential roles in cell adhesion and cell signalling. APP is one of the most abundant proteins in the brain and therefore there was initial shock when APP knock-out mice showed little phenotype (Muller et al., 1994, Zheng et al., 1995). $APP^{-/-}$ mice are viable and fertile with a subtle phenotype. They show reduced locomotor activity, reduced body and brain weight, reactive gliosis, and hypersensitivity to kainate induced seizures (Zheng et al., 1995, Steinbach et al., 1998). However, when APP/Amyloid precursor-like protein 2 (APLP2), APLP1/APLP2 and APP/APLP1/APLP2 combined knockouts were made, these resulted in early postnatal lethality. These knock-outs revealed the APP family play a role in development of the nervous system; specifically synapse structure and function, neuronal migration and adhesion (von Koch et al., 1997, Heber et al., 2000, Herms et al., 2004).

## 1.11.1 Full length APP

Due to its structural and processing similarities to receptor proteins such as notch, APP has been proposed to act as a cell surface receptor (Wolfe and Guenette, 2007, Muller and Zheng, 2012). The ability of the APP family to form both homo- and hetero-dimers intercellularly through their E1 domains, and their interactions with extracellular matrix proteins such as collagen and laminin (Kibbey et al., 1993, Beher et al., 1996, Caceres and Brandan, 1997), led to the proposal that they may play a role in cellular adhesion, possibly localised to the synapse (Soba et al., 2005).

## 1.11.2 Soluble ectodomains

The initial secretase cleavage event causes release of soluble ectodomains of APP (sAPP), sAPPα is released via α-secretase-mediated APP cleavage whilst sAPPβ, which is slightly smaller, is released via β- secretase-mediated APP cleavage. Ring et al (2007) showed that all phenotypes of APP deficient mice could be rescued by sAPPα knock-in, suggesting that this is the physiologically important component of APP. sAPPα has been shown to be required for LTP, a crucial mechanism in cognition and memory (Taylor et al., 2008), supported by findings that intracerebral sAPPα injections enhance memory performance in adult rats (Gralle and Ferreira, 2007). sAPPα is also neurotrophic and neuroprotective under stressed conditions (Mattson et al., 1993, Gralle and Ferreira, 2007). Application of sAPPα to neural progenitor cells led to increased proliferation both *in vitro* and *in vivo* in the adult mouse CNS, suggesting a similar role to notch in adult stem cell regulation (Caille et al., 2004, Demars et al., 2011).

In contrast, sAPPβ although highly stable and abundant, does not rescue APP knockout phenotype (Li et al., 2010), thus highlighting the importance of regulation of APP processing to maintain appropriate levels of these different functionalities. Furthermore, unlike sAPPα, sAPPβ can be further processed by caspases and bind death receptor 6 (DR6), which is associated with axonal pruning and degeneration (Nikolaev et al., 2009). This suggests sAPPβ plays a physiological role in the selective neuronal loss required during development, but in adults could also be part of the pathological process of neuronal degeneration seen in AD.

## 1.11.3 AICD

The AICD is produced by ε cleavage of APP by γ-secretase. Through its binding partners the AICD has been implicated in a number of APP-dependent functions, including synaptic activity (King and Scott Turner, 2004, Ghosal and Pimplikar, 2011) and axonal guidance (Guenette et al., 2006). Due to the short half-life of AICD in the

cytoplasm it is difficult to study its biochemical features and physiological functions. The AICD contains three phosphorylation sites including the main regulatory site, Thr668. Phosphorylation at this site is neuron specific and therefore thought to be central to the physiological function of APP. It regulates interactions with binding partners such as Fe65 and may facilitate BACE1 processing and the subsequent γ-secretase cleavage of the C99 stub (Lee et al., 2003, Chang et al., 2006, Mazzitelli et al., 2011).

Alterations to cellular levels of AICD through γ-secretase inhibition or increased AICD degradation caused decreased $Ca^{2+}$ stores in the ER, reduced ATP content and disrupted mitochondrial function. This suggests that AICD plays a role in calcium homeostasis of neurons, which is critical for neuronal activity (Hamid et al., 2007).

The transcriptional activity of the AICD is still a hotly contested debate. Cao and Sudhof (2001) reported that AICD could form a transcriptionally active complex with Fe65 and Tip60, however it was later shown that Fe65 could induce transcription outside of an AICD complex, following AICD-dependent modification of its structure (Cao and Sudhof, 2004). Despite these uncertainties as to the dominant transcriptional molecule, the putative target genes suggest a role for AICD in regulation of APP processing, with an elaborate feedback loop involving APP itself and enzymes involved in its degradation (Beckett et al., 2011).

AICD can be cleaved by caspases, which are traditionally associated with apoptotic cell death (Bertrand et al., 2001, Madeira et al., 2005). This may indicate the AICD plays a similar role to that suggested for sAPPβ during development in mediating selective neuronal loss. However, caspases have cellular roles other than mediation of apoptosis, although less well characterised. Caspase 3 activation has been shown to induce changes in growth cone direction, which could indicate a role in the axonal guidance function of APP or caspase 1 can affect synaptic plasticity (Campbell and Holt, 2003, Lu et al., 2006). As the identity of the caspase(s) responsible for AICD cleavage remains uncertain, these roles are speculative.

## 1.11.4 Amyloid β

Much of the work on Aβ has focussed on its role as the aggregation prone, neurotoxic species that interferes with synaptic function in affected neurons (Walsh et al., 2002, Lesné et al., 2006, Li et al., 2009, Wu et al., 2010), however it has also been shown to have neurotrophic and signalling functions (Yankner et al., 1990, Kamenetz et al., 2003). Low picomolar concentrations of Aβ monomers and oligomers, including the more hydrophobic, aggregation prone $A\beta_{42}$, cause an increase in LTP mediated by α7 nicotinic acetylcholine receptors, associated with improved cognition (Puzzo et al., 2008). Further to this, $A\beta_{42}$ monomers are able to support survival of developing primary neurons in the absence of trophic factors. They also protect mature neurons against excitotoxic death through a mechanism involving phosphatidylinositol 3-kinase (PI3K) signalling and IGF1 receptors (Giuffrida et al., 2009).

Misregulation and overproduction of Aβ have a toxic, gain of function phenotype at the neuronal synapse. Aβ can form dimers, trimers and low n-number soluble oligomers. These can then go on to form insoluble protofibrils and eventually the fibrils seen in amyloid plaque pathology. Initial *in vitro* tests suggested that the insoluble fibrils were neurotoxic. This was not supported by parallel work in post-mortem brains showing amyloid pathology had a weak correlation with the severity of dementia and that there was a better correlation with soluble forms of Aβ. Small $A\beta_{42}$ oligomers were able to kill mature neurons at nanomolar concentrations (Lambert et al., 1998), with initial Aβ mediated inhibition of synaptic LTP long before the cellular degeneration. Although direct neurotoxicity of Aβ remains controversial, oligomers of Aβ secreted from primary neurons were shown to inhibit LTP *in vivo* in rats and this was abrogated by IDE breakdown or immunodepletion of Aβ from the medium, confirming Aβ-mediated synaptic dysfunction (Walsh et al., 2002).

A number of studies have suggested that Aβ mediates its effect through disruption of trafficking of key receptor subunits. Low nanomolar Aβ increased trafficking of

NR2B subunits which led to down-regulation of extracellular signal-regulated kinase (ERK) and cAMP response element binding protein (CREB)-mediated pathways and disruption of LTP (Li et al., 2011). Another study showed Aβ oligomers reduced GluA1 phosphorylation, crucial for its transport, this reduction in phosphorylation was shown to be due to Aβ-mediated changes in $Ca^{2+}$ influx (Minano-Molina et al., 2011).

As well as Aβ being able to modulate synaptic activity, synaptic activity is also able to regulate Aβ. Physiological changes in neuronal activity are sufficient to dynamically modulate ISF Aβ concentrations, which themselves are correlated with plaque growth *in vivo* (Bero et al., 2011). Neuronal activity also regulates the equilibrium between the amyloidogenic and the non-amyloidogenic pathway. Synaptic glutamate release causes a shift away from Aβ production. Conversely, extrasynaptic release and high levels of glutamate release increase Aβ levels (Hoey et al., 2009, Bordji et al., 2010, Verges et al., 2011).

These conflicting functions of Aβ suggest it plays a physiological role in maintenance of synaptic activity, promoting LTP at low concentrations and inhibiting it at higher concentrations.

## 1.12 Flavonoids and AD risk

An increasing body of evidence suggests that a group of plant-derived compounds, the flavonoids, might be effective in preventing the onset and/or slowing progression of AD (Williams and Spencer, 2012). Due to the prolonged failure of conventional pharmaceutical drug development to bring AD modifying therapies to market, there is considerable interest in alternative strategies to reduce the burden of the disease.

As secondary metabolites present in fruits and vegetables, flavonoids are present in human diets, with a mean daily intake of approximately 14 mg (Commenges et al., 2000, Letenneur et al., 2007). There is great variability in flavonoid bioavailability; many are directly excreted but it has been shown that select flavonoids and their

metabolites are detectable in the bloodstream hours after ingestion (Ramassamy, 2006). The extensive metabolism of flavonoids following absorption and the lack of bioavailability of many flavonoids has proved a significant hurdle in the study of their reported beneficial effects in AD. However a number of studies have now been conducted suggesting flavonoids hold promise in the prevention of AD.

### 1.12.1 Flavonoid structures

Flavonoids are the largest group of polyphenolic compounds and have several thousand known members (Ramassamy, 2006). They occur ubiquitously in plant-derived foods with the major dietary sources being fruits, vegetables and beverages such as tea and red wine. In plants, they are synthesised from phenylalanine through either the phenylpropanoid or the acetate-malonate pathways in a series of diverse, plant specific reactions to generate molecules with the same central structure but many different substitutions (Weston and Mathesius, 2013).

All flavonoids have the same basic structure of two aromatic rings (A and B) bound together by 3 carbons forming an oxygenated heterocycle (C) (Spencer, 2010) (Figure 1.9). Flavonoids can be divided into 6 subclasses dependent on the type of heterocycle present in the molecule, these are: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (Manach, 2004) (Figure 1.9).

The flavonols are the most abundant subclass of flavonoid, the most prevalent molecules being kaempferol and quercetin, and are rich in foods such as broccoli and curly kale. They are distinguished by the presence of a 3-hydroxy-pyran-4-one C ring (Figure 1.9). The flavones, such as apigenin and luteolin, are rich in celery and cereal grains. These lack the 3 hydroxyl substitution on the C ring (Figure 1.9). Isoflavones, such as genistein and daidzein, are rich in soy products. They have their B ring located at the 3 position on the C ring (Figure 1.9). Flavanones, such as naringenin and hesperetin, are rich in citrus fruits and are lacking the 2, 3 double bond and 3-hydroxyl on the C ring (Figure 1.9). Flavanols such as (-) epicatechin, are rich in chocolate and green tea and are lacking the 2, 3 double bond.

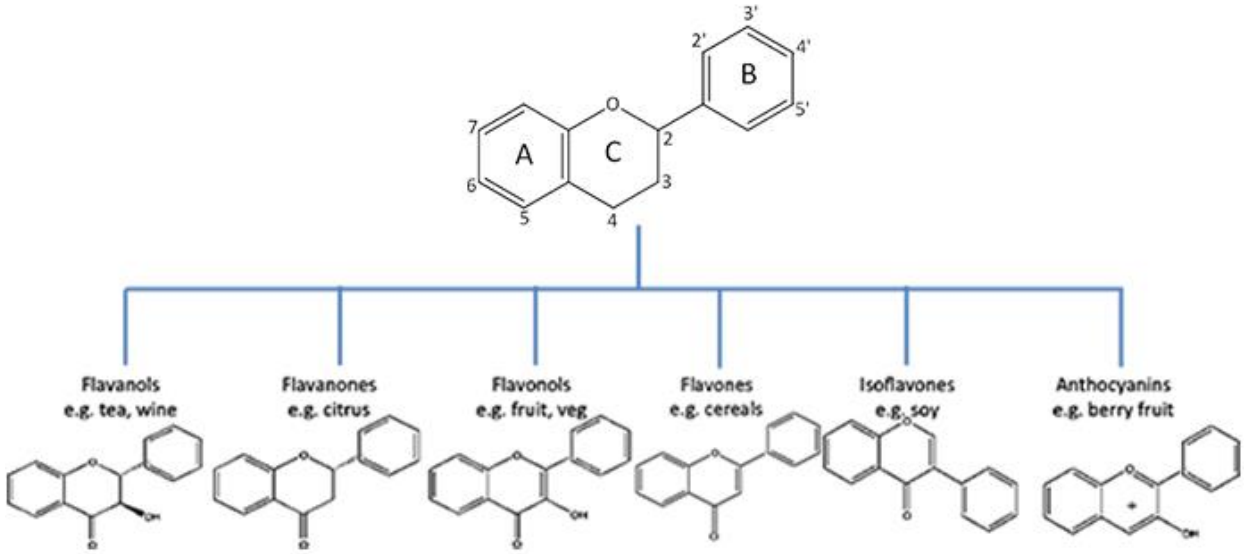



Figure 1.9: **Flavonoid structures and families**

All flavonoids have the same basic structure: 2 aromatic rings (A and B) and an oxygenated heterocycle (C). Flavonoids are categorised according to the type of heterocycle present into flavonols, flavones, isoflavones, flavanones, flavanols and anthocyanidins. Flavonoids from each group are prevalent in different foodstuffs. Flavonoids within a sub-family are categorised according to substitutions on the aromatic rings.

Anthocyanidins, such as cyanidin and pelargonidin, are rich in berries, and are lacking the 2, 3 double bond and the 4-one group (Rice-Evans et al., 1996, Manach, 2004) (Figure 1.9). Flavanols can also exist as polymers, known as proanthocyanidins, which vary based on the constitutive monomers and linkages.

### 1.12.2 Epidemiological evidence of efficacy

Epidemiological evidence suggests that regular intake of flavonoid-rich foods, such as berries and green tea, may result in cognitive benefits (Kuriyama et al., 2006, Letenneur et al., 2007, Devore et al., 2012) (Table 1.2). The Personnes âgées quid (Paquid) and Nurses' Health study, two independent population studies focussing on the health of ageing populations have shown that consumption of flavonoid-rich foods resulted in improved cognition in the elderly. The Paquid study carried out dietary assessments four times over a ten year period in people over sixty-five at base line concurrent with cognitive ability assessments. They showed a decreased rate of decline in MMSE score associated with increased flavonoid intake (Letenneur et al., 2007). The American Nurses' Health study of retired nurses over seventy years old, carried out semi-quantitative dietary assessments and cognitive performance assessments over a four year period. Results showed that increased flavonoid intake, especially berries, resulted in a two and a half year mean difference in relative years of cognitive decline (Devore et al., 2012). The Tsurugaya Project cross-sectional study supported these prospective analyses: in Japanese subjects over seventy years old, increased flavonoid-rich green tea consumption had an inverse dose-response relationship with cognitive impairment, defined as an MMSE score less than twenty-six (Kuriyama et al., 2006).

Studies more specifically focussed on dementia suggested that higher flavonoid intake was inversely related to risk (Commenges et al., 2000, Dai et al., 2006, Barberger-Gateau et al., 2007) (Table 1.2). As part of the Paquid population study, cognition in 1367 over sixty-five year olds was measured every two years from 1991-96. Estimations of total flavonoid intake from wine, tea, fruits and vegetables were calculated for the same time period and incident cases of dementia observed.

| Study | Foodstuff | Age of subjects | Length of study (years) | Methodology | Outcomes | Reference |
|---|---|---|---|---|---|---|
| **Paquid (French)** | Flavonoid intake | 65+ | 5 | Questionnaires | Intake of flavonoids inversely related to risk of dementia incidence | (Commenges et al., 2000) |
| **Honolulu-Asia Aging study** | Antioxidants | 45-68<br>70+ | 6 | 24h dietary recall<br>5 psychometric tests | Midlife dietary intake of antioxidants doesn't modify risk of late life dementia. | (Laurin et al., 2004) |
| **Kame Project (Japanese Americans)** | Fruit and Vegetable juices | 65+ | 7-9 | Interviews; questionnaires every 2 years | Consumption of juices associated with decreased risk of developing AD<br>effects larger with ε4 allele | (Dai et al., 2006) |
| **Tsurugaya project (Japanese)** | Green tea | 70+ | Geriatric assessment in 2002 | Questionnaires<br>1 psychometric test | Cognitive impairment decreased with increasing tea consumption | (Kuriyama et al., 2006) |
| **Three city cohort (French)** | Fruit and vegetables | 65+ | 4 | Questionnaire | Daily fruit and veg consumption assoc. with decreased risk of dementia | (Barberger-Gateau et al., 2007) |
| **Paquid (French)** | Flavonoid intake | 65+ | 10 | 3 psychometric tests<br>4 sets over 10 years | Flavonoid intake associated with better cognitive performance at baseline & better evolution over time. | (Letenneur et al., 2007) |
| **Three city cohort (French)** | Fruit and vegetables | 65+ | 5 | 4 psychometric tests | Better adherence to Mediterranean diet associated with slower MMSE decline | (Feart et al., 2009) |
| **Nurses' Health Study (American)** | Blueberries & Strawberries | 70+ | 6 years | Questionnaires<br>6 psychometric tests | Women with higher berry intake delayed cognitive aging | (Devore et al., 2012) |

Table 1.2: **Epidemiological evidence of flavonoid efficacy**

Adjusted relative risk of dementia in those with the highest intakes of flavonoids was significantly lower than those with the lowest intake of flavonoids (Commenges et al., 2000). Following these results, the same group looked in a different French population. The Three-City Cohort conducted in Bordeaux, Dijon and Montpellier measured the incidence of AD over four years and correlated this with daily intake of fruits and vegetables. Once again this concluded that there was a reduced risk of incident AD following regular intake of fruit and vegetables (Barberger-Gateau et al., 2007). In separate work, the Kame project, a study of aged (mean 71.8 years) Japanese-Americans living in Washington, USA, showed that consumption of three or more fruit or vegetable juices per week decreased the risk of developing AD compared to drinking less than one juice per week. This effect was enhanced in people carrying the Apoε4 allele and in those less physically active (Dai et al., 2006). These promising observations have led to significant interest in identifying the underlying mechanisms through which flavonoids exert their beneficial effects and which specific flavonoids are conveying the benefits.

### 1.12.3 *In vivo* flavonoid studies

Due to the promising data from epidemiological studies, many studies have investigated the impact of flavonoid treatment on transgenic models of AD in attempts to understand the mechanisms through which flavonoid-rich diets are reducing the risk of AD. These have suggested select flavonoids can have positive effects on a number of AD-related outcomes including amyloid pathology, toxic APP metabolite levels, and memory deficits (Williams and Spencer, 2012).

According to the amyloid cascade hypothesis, APP metabolism and its misregulation are the most direct targets for flavonoids to impact on disease progression (Hardy and Higgins, 1992). Rodent models of AD, where pathological APP processing has been induced have been utilised to study the effect of flavonoid treatment on amyloid pathology. Flavanols have shown particular efficacy in reducing Aβ burden: three flavanols epigallocatechin gallate (EGCG), tannic acid and a grape seed

polyphenol extract reduced Aβ immunoreactive deposits and EGCG additionally reduced thioflavin S-positive Aβ deposits, indicating reduced Aβ plaques (Rezai-Zadeh, 2005, Rezai-Zadeh et al., 2008, Wang et al., 2008, Mori et al., 2012). This activity is not unique to the flavanols however, curcumin via gavage and intraperitoneal (IP) injection reduced plaque burden in the hippocampus and cortical areas (Yang et al., 2005, Garcia-Alloza et al., 2007).

APP metabolite levels following chronic flavonoid treatment show consistent changes using a range of individual flavonoids and different methodologies. Reductions in soluble Aβ levels were evident following sixty-day and four-month IP injection with EGCG and nobiletin respectively, following ten months of curcumin and myricetin via gavage and following five months of monomeric catechins in drinking water (Rezai-Zadeh, 2005, Onozuka et al., 2008, Hamaguchi et al., 2009, Wang et al., 2012). Additionally, EGCG and a Cabernet Sauvignon extract increased sAPPα levels following chronic IP injection and oral delivery in aged Tg2576 mice respectively (Rezai-Zadeh, 2005, Wang et al., 2006, Rezai-Zadeh et al., 2008).

AD is characterised by the gradual deterioration of mental faculties. Most of the epidemiological evidence for flavonoid efficacy was based on cognitive test score comparisons. Therefore, several *in vivo* studies of flavonoids have focussed on their proposed ability to affect memory. In wild type (WT) mice, chronic (-) epicatechin treatment via gavage and in drinking water improved latency in the standard MWM test of spatial memory (van Praag et al., 2007). Further studies have been carried out on an AD background. Monomeric grapeseed polyphenols (main constituent (-) epicatechin), and a grape seed polyphenolic extract (GSPE) reduced latency in female Tg2576 mice in the MWM test (Wang et al., 2008, Wang et al., 2012). Green tea catechins reduced errors in the radial maze, a test of working and reference memory, following cerebral Aβ injections in Wistar rats (Haque et al., 2006, Haque et al., 2008). Cabernet Sauvignon treatment led to reduced latency of escape in Tg2576 mice following training in the Barnes' maze, a test of spatial learning and memory (Wang et al., 2006).

Taken together, these *in vivo* studies suggest flavonoids are able to impact on brain Aβ pathology in a variety of models and that flavonoid treatment can lead to

improvements in cognitive deficits associated with AD. Despite these positive results, these studies did not elucidate the cellular mechanism(s) through which flavonoids are able to exert these effects.

## 1.12.4 Mechanism of action of flavonoids

It has mainly been through *in vitro* studies that clues as to the underlying mechanism of action of flavonoids have been discovered. The findings of these segregate into several distinct biological processes: actions as antioxidant molecules, interactions with APP and its processing machinery and interactions with neural signalling pathways. The specific mechanisms through which flavonoids may affect risk, delay onset or slow progression of AD have been particularly intensively examined.

### *1.12.4.1 Flavonoids as antioxidant molecules and AD risk*

Due to their chemical structure, initially flavonoids were predicted to act as antioxidants. Oxidative stress is defined as a disturbance between the pro-oxidant and antioxidant systems in favour of the former (Ben Mahdi et al., 2000). Pro-oxidant systems, particularly ATP generation in the brain, give rise reactive oxygen species (ROS) including superoxide, hydroxyl radicals and hydrogen peroxide ($H_2O_2$). These are neutralised by antioxidant enzymes such as superoxide dismustase (SOD-1), heme oxygenase-1 (HO-1), catalase and glutathione, maintaining the redox state of the cell (Petersen et al., 2007).

The CNS is particularly vulnerable to oxidative stress due to its high oxygen consumption rate, high lipid content, and the relatively low levels of endogenous antioxidant enzymes compared to other tissues (Markesbery, 1997). AD brains exhibit evidence of increased oxidative stress including increased lipid peroxidation, increased protein and DNA oxidation, increased levels of advanced glycation end products (AGE) and increased iron levels (Smith et al., 2000, Wang et al., 2013). The pathology of AD is increasingly being associated with oxidative stress, such as the

presence of glycated proteins in amyloid plaques, and evidence suggesting Aβ oligomers generate $H_2O_2$ and hydroxyl radicals (Kapogiannis and Mattson, 2011). These findings implicate the use of antioxidants as a potential treatment strategy.

Flavonoids have been proposed to act as antioxidants through several mechanisms, as reducing agents, hydrogen donating antioxidants or as singlet oxygen quenchers (Rice-Evans et al., 1996) (Figure 1.10(A)). Studies measuring their antioxidant capacity have shown them to be more effective per mole than the traditional antioxidants such as ascorbate (Rice-Evans et al., 1996, Pietta, 2000). The rate of reaction of flavonoids with free radicals and the subsequent stability of the reduced free radical and flavonoid radical also provides a strong basis for them to act as antioxidants *in vivo* (Bors et al., 1990). The structural basis of the flavonoids' antioxidant effects were dissected and highlighted the hydroxyl groups on the B ring as the most significant determinant of scavenging ability (Heim et al., 2002). These increase delocalisation of electrons in the phenol ring, allowing greater stability of the flavonoid radical and therefore greater hydrogen donating capabilities. The double bond at the 2, 3 position in concert with the 4-one functional group and the 3' and 5 hydroxyl of the A and C rings are also required for maximal scavenging potential. The 2, 3 double bond is thought to be especially critical for the lipid peroxidation inhibition activities of flavonoids (Rice-Evans et al., 1996).

However, following ingestion flavonoids are heavily metabolised in the liver and small intestine, often methylated and glucuronidated. Additionally, glycosides are often the natural forms of flavonoids found in the human diet (RiceEvans and Miller, 1997). These additional groups create steric hindrance and alter the planarity of the molecule, reducing the antioxidant capacity significantly. Therefore, orally administered flavonoids probably only serve as antioxidants in the gastrointestinal tract (Jovanovic et al., 1998) Availability studies have also shown that levels of flavonoids entering the brain are in the nanomolar range(van Praag et al., 2007, Wang et al., 2012). The high concentrations required to have an additive effect over endogenous brain antioxidants, makes it is unlikely that this is their mechanism of action.

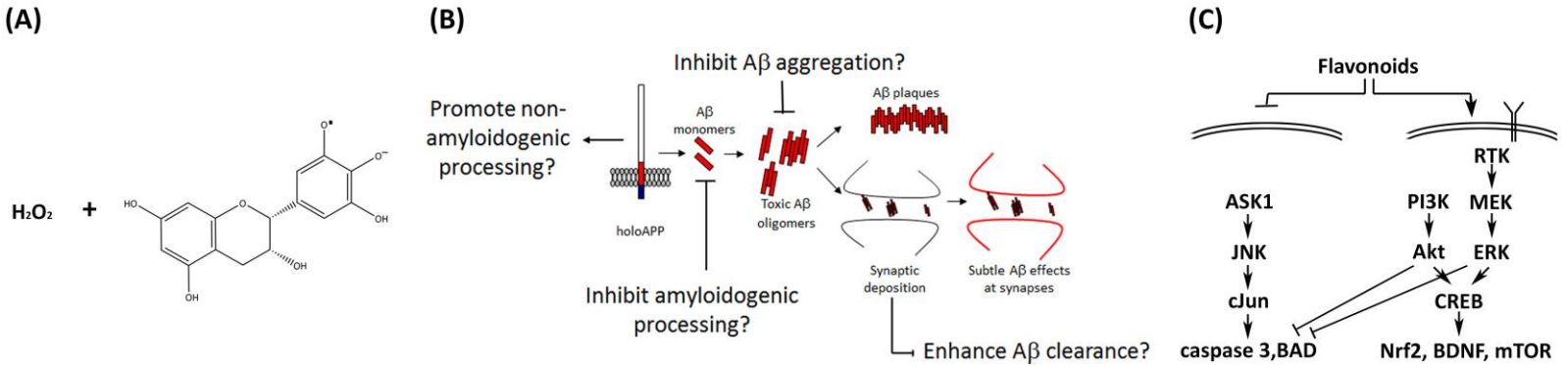



Figure 1.10: **Potential mechanisms of flavonoid-mediated effects in AD**

(A) Flavonoids as antioxidants. Due to their structure, *in vitro*, flavonoids can act as hydrogen donators, singlet oxygen quenchers and as reducing agents. (B) Flavonoid modulation of Aβ pathology. They can increase α-secretase activity or inhibit β secretase activity. They can alter the aggregation of Aβ into less toxic structures or inhibit fibrillisation. They can enhance Aβ clearance through improved blood flow. (C) Flavonoid modulation of neuronal signalling pathways involved in cell survival and cognitive performance. Flavonoids activate the ERK-CREB and PI3K-mTOR pathways leading to changes in LTP and cognitive performance. They also inhibit pro-apoptotic signalling through inhibition of JNK, promoting neuronal survival.

### *1.12.4.2 Flavonoids as APP metabolism modulators*

Another possible mechanism that has been investigated is through direct interaction of flavonoids with APP, its metabolites and proteolytic machinery. Flavonoids from across the families are effective at reducing Aβ levels (Rezai-Zadeh, 2005, Obregon et al., 2006, Wang et al., 2006, Lin et al., 2009, Ho et al., 2013). Flavonoids have been shown to achieve this through different mechanisms (Figure 1.10(B)). EGCG was shown to increase sAPPα production (Giunta et al., 2010) through maturation of the α secretase enzyme ADAM10 via PKC phosphorylation (Levites et al., 2003, Obregon et al., 2006) possibly through estrogen receptor α (ERα) activation (Fernandez et al., 2010). This favoured non-amyloidogenic processing and precluded formation of Aβ. Treatment of Tg2576 primary neurons with Cabernet Sauvignon, which has a high flavanol content and treatment with (-) epicatechin, showed reduced Aβ production were also due to increased non-amyloidogenic metabolite production (Rezai-Zadeh, 2005, Wang et al., 2006).

The β secretase enzyme BACE1 has also been identified as a potential site of interaction, select flavones and flavonols are able to reduce BACE1 activity in primary neurons, and *in silico* docking studies predict catalytic domain binding sites (Shimmyo et al., 2008b). Furthermore, Aβ-induced BACE1 up-regulation can be attenuated by EGCG and curcumin treatment (Shimmyo et al., 2008a).

Although the production of Aβ from APP is central to AD pathogenesis, it is the oligomerisation and fibrillogenesis of these peptides that is thought to confer the associated neurotoxic effect (Walsh and Selkoe, 2007). Studies show that flavonoids are capable of reducing production of high molecular weight Aβ oligomers and fibrillogenesis (Ono et al., 2003, Ehrnhoefer et al., 2008, Wang et al., 2008, Ho et al., 2013). However, all of these studies were conducted with synthetic Aβ peptides mixed with micromolar concentrations of flavonoid, and so although promising observations, the biological relevance of these interactions remains to be explored.

### *1.12.4.3 Flavonoids as cell signalling modulators*

Away from direct effects on APP metabolism, flavonoids are also thought to be neuroprotective through activation of pro-survival pathways. Active MAPK pathways act as modulators for neuronal survival, regeneration and death. Activation of the ERK pathway has generally been associated with cell survival (Schroeter et al., 2002). Nanomolar concentrations of flavonoids, in particular flavanols, activate the ERK pathway through phosphorylation of ERK1/2 (Schroeter et al., 2001, Vauzour et al., 2007, Rainey-Smith et al., 2008) in a mitogen activated ERK kinase (MEK) dependent manner (Schroeter et al., 2007), possibly through activation of cell surface tyrosine receptors such as tropomyosin receptor kinase B (TrkB) (Jang et al., 2010). This results in downstream phosphorylation of CREB (Maher et al., 2006, Ho et al., 2013) which can then lead to up-regulation of signalling pathway genes such as those associated with increased basal synaptic transmission (Schroeter et al., 2007, Wang et al., 2012). This then leads to improvements in cognition such as the significant improvement in object recognition test seen with oral fisetin treatment in mouse (Maher et al., 2006) (Figure 1.10(C)).

PI3K has been implicated in synaptic plasticity and as a mediator of the oxidative stress response (Schroeter et al., 2002), making it a central decision maker between neuronal survival and cell death (Figure 1.10(C)). Studies suggest these key decisions can be influenced by select flavonoids. Most compellingly, one of the most specific PI3K inhibitors, LY294002, is based on the chemical structure of the flavone quercetin, predicted to interact directly with the ATP binding domain of the kinase (Vlahos et al., 1994). Epicatechin-mediated up-regulation of glutathione has also been shown to be PI3K dependent (Bahia et al., 2008). Many of the flavonoid-dependent effects mediated by ERK also have a PI3K/pAkt component. Hesperetin has been shown to have anti-apoptotic effects following $H_2O_2$ exposure, and this is mediated by phosphorylation of Akt and ERK leading to increased bcl-2-associated death promoter protein (BAD) phosphorylation (Vauzour et al., 2007).

Finally, the nuclear pool of mammalian abelson murine leukaemia viral oncogene homolog protein (c-Abl) has been implicated in neuronal cell death and is enhanced in Swedish mutant APP (APPswe) overexpressing cell lines. Nuclear levels of c-Abl were modulated by the flavanol EGCG, which attenuated increased APPswe expression and prevented binding of the normal APP nuclear trafficking partner, Fe65, thus enhancing neuronal survival (Lin et al., 2009).

A wide range of mechanisms have been proposed for how flavonoids exert their beneficial effects and it may be that they are not mutually exclusive. Flavonoids may be able to mediate a number of different effects in a single cell. They may impact on general cell health through survival signalling pathways, and in parallel impact on the specific processing pathway of APP to address its misregulation.

## 1.13 Hypothesis and Aims

The **hypothesis** of this thesis was that flavonoids would inhibit amyloidogenic APP processing in primary cortical neurons.

To investigate this hypothesis, a number of specific **aims** were defined:

To characterise the APP-Gal4 assay as a measure of amyloidogenic APP processing in primary neurons.

To screen a library of flavonoids for inhibitory actions on APP processing in primary neurons.

To define the underlying cellular mechanisms through which effective flavonoids were inhibiting amyloidogenic APP processing.

# Chapter 2

## 2. Materials and Methods

# 2.1. Materials

All bench chemicals were analytical grade, purchased from Sigma-Aldrich (Poole, UK), Fisher Scientific (Loughborough, UK) or VWR (Lutterworth, UK). Cell culture reagents were purchased from Invitrogen (Paisley, UK). $A\beta_{x-40}$ ELISA kits (Cat No. SIG-38954) were purchased from Covance (Princeton, USA). pFR-luciferase and TK-Renilla plasmids used in luciferase assays were purchased from Promega (Southampton, UK).

## 2.1.1. Compounds

Flavonoids were purchased from Extrasynthese (Genay Cedex, France). N-[n-(3, 5-difluorophenactetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT), PD184352, UO126, NMDA and (s) - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were purchased from Tocris (Bristol, UK). TAPI-1 was purchased from Enzo Life Sciences (Exeter, UK). Amyloid Precursor Protein β-Secretase Inhibitor (BsI) and β-secretase Inhibitor IV (BiV) were purchased from Calbiochem (Merck Chemicals, Nottingham, UK). Complete Protease Inhibitor Cocktail tablets and PhosSTOP Phosphatase Inhibitor Cocktail tablets were purchased from Roche Applied Science (Burgess Hill, UK). Recombinant human BDNF was purchased from Peprotech (London, UK). 4', 6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (Paisley, UK).TAPI, DAPT, BsI and BIV were made up as 1000x concentrated stocks in DMSO. Flavonoids were made up as 10 mM stocks in methanol. All other compounds were made up as 1000x concentrated stocks in ultrapure water. (See Table 2.1 and Table 2.2 for further details).

| Treatment | Details | Source | Stock concentration | Solvent | Diluent | Extra information |
|---|---|---|---|---|---|---|
| **NMDA** | Selective NMDA agonist | Tocris | 10 mM | $dH_2O$ | N/A | Added directly to culture medium |
| **(s)-AMPA** | Selective AMPA agonist | Tocris | 10 mM | $dH_2O$ | N/A | Added directly to culture medium |
| **UO126** | Selective, non-competitive inhibitor of MEK1/2 | Tocris | 10 mM | DMSO | N/A | Added directly to culture medium |
| **PD184352** | Selective, non-competitive inhibitor of MEK1/2 | Tocris | 2 mM | DMSO | N/A | Added directly to culture medium |
| **BDNF** | Recombinant human BDNF | Peprotech | 50 µg/mL | $dH_2O$ | $dH_2O$ | Diluted to 100x required. Added directly to culture medium |
| **Flavonoids** | Analytical grade plant derived compounds (≥ 99% purity) | Extrasynthese | 10 mM | Methanol | N/A | Added directly to culture medium |

Table 2.1: **Modulators of synaptic signalling pathways**

| Treatment | Details | Source | Stock concentration | Solvent | Diluent | Extra information |
|---|---|---|---|---|---|---|
| **TAPI** | Metalloproteinase inhibitor | Enzo life sciences | 50 mM | DMSO | N/A | Added directly to culture medium |
| **BsI** | BACE1 inhibitor | Calbiochem | 10 mM | DMSO | N/A | Added directly to culture medium |
| **BiV** | BACE1 inhibitor | Calbiochem | 10 mM | DMSO | N/A | Added directly to culture medium |
| **DAPT** | γ secretase inhibitor | Tocris | 10 mM | DMSO | N/A | Added directly to culture medium |

Table 2.2: **Secretase enzyme modulators**

## 2.1.2. Antibodies

ERK1/ERK2 (p42/44, T202/Y204) rabbit polyclonal antibody was purchased from Cell Signalling Technology (Massachusetts, USA). ERK2 (C14) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (California, USA). MAP2 rabbit polyclonal antibody, ADAM10 (735-749) rabbit polyclonal and APP (22C11) mouse monoclonal antibody were purchased from Millipore Bioscience Research Reagents (California, USA). Tau mouse monoclonal antibody was purchased from Chemicon (Merck Millipore, California, USA). β-tubulin III mouse monoclonal antibody, β actin (AC-74) mouse monoclonal antibody and BACE1 (2C13) mouse monoclonal antibody were purchased from Sigma (Poole, UK). sAPPα (5G11) rat monoclonal antibody, specific for murine sAPPα in culture media, generated by immunisation against DAEFGHDSGFEVRHQKC-COOH peptide; sAPPα 14D6, specific for human sAPPα in culture media, generated by immunization against CYEVHHQ-COOH peptide; sAPPβ (192WT) rabbit polyclonal, specific for sAPPβ in culture media (all provided by S. Lichtenthaler).

## 2.1.3. Plasmids and Viruses

pRC-CMV-$APP_{695}$-Gal4 and pRC-CMV-$APP_{695}$-Gal4Δ were as described previously (Hoey et al., 2009). pRC-CMV vector containing a cDNA encoding for the Swedish familial mutation of human APP695 fused in-frame at its C-terminus to the Gal4 yeast transcription factor was created using pRC-$APP_{695}$-Gal4 plasmid (see Appendix 1) and introduction of a double point mutation at positions K595N/M596L of $APP_{695}$ using the QuikChange XL Site-Directed Mutagenesis kit according to the manufacturer's instructions (Stratagene). pFR-Luciferase reporter vector with Firefly (*Photinus pyralis*) luciferase gene under the control of a synthetic promoter containing 5 tandem repeats of the yeast Gal4 upstream activation sequence (UAS) upstream of a minimal TATA box and phRL thymidine kinase (TK) vector containing sea pansy (*Renilla reniformis*) luciferase gene under control of the herpes simplex virus (HSV)–TK promoter, from Promega. pC1-CMV vector containing cDNA

encoding human Fe65 has been described previously (Perkinton et al., 2004). pSecTag2 vector containing cDNA encoding for human notch3 fused in frame at its C terminus to the yeast transcription factor Gal4 was kindly provided by Dr Michael Perkinton (King's College, London) (see Appendix 2). The adenovirus vector Ad5-Egr1-Luciferase containing cDNA for luciferase under the control of the murine Egr1 promoter was kindly provided by Dr C. Caunt. (Department of Biology and Biochemistry, University of Bath, Bath, UK). This was generated by subcloning an Egr-1 promoter Sal1 fragment into an Xho1 digest of pAd5-luciferase. Viral generation from this shuttle vector has been previously described (Caunt et al., 2008) (see Appendix 3).

#### *2.1.3.1. Large Scale Plasmid Purification*

QIAgen Hi Speed Maxi kits were used according to the manufacturer's instructions. Bacterial glycerol stocks were cultured over day in 5 ml Luria-Bertani (LB) broth, 250 µL were then transferred to 250 mL LB and cultured overnight. The culture was pelleted (6000x g for 15 minutes at 4$^{o}$C) and completely resuspended by pipetting. Cells were then lysed and filtered using QIAfilter Maxi Cartridge. Cell supernatant was washed, eluted and precipitated in isopropanol (0.7 x volumes). The eluate/isopropanol mixture was passed through a QIAprecipitator, collecting the DNA. DNA was washed with 70% ethanol and eluted with UltraPure water (Promega). DNA quantified by Nanodrop spectrophotometer.

# 2.2. Methods

## 2.2.1. Primary neuronal culture

Nunc tissue culture plates of appropriate size for experimental design were coated overnight with 20 µg/mL poly-D-lysine (Sigma) dissolved in tissue culture standard water (Gibco, Invitrogen). Plates were then washed with 1/2 well volume tissue culture standard water, 1x PBS, then Neurobasal wash media (Phenol Red free Neurobasal, 60 µg/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-glutamine, 10% foetal calf serum (Invitrogen).

CD1 or C57/BL6 pregnant mice were sacrificed by cervical dislocation at gestation day 15-16. Embryos were removed and placed in PBS ($Ca^{2+}$ and $Mg^{2+}$ free PBS, Invitrogen). Embryos were kept in PBS throughout the dissection. Embryonic cortices were dissected, striatal regions and meninges removed.

Cortices were mechanically dissociated using a fire polished glass pipette pre-coated with heat inactivated foetal calf serum. The homogeneous cell suspension was centrifuged at 500xg for 5 min at RT. The resultant pellet was resuspended in Neurobasal media (Phenol Red free Neurobasal, 60 µg/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-glutamine (Invitrogen)) and plated out at required densities for experimental design, stated in individual methodologies. Neurons were incubated at 37$^{o}$C in a humidified atmosphere (95% air and 5% $CO_2$), and were used between 5 and 10 days *in vitro* (DIV).

## 2.2.2. Cell culture

HEK293T cells were cultured in 10% FCS, 60 µg/mL penicillin, 100 µg/mL streptomycin supplemented Dulbecco's modified Eagle's medium (DMEM) Glutamax High glucose (Gibco). SH-SY5Y cells were cultured in 15% FCS, 60 µg/mL

penicillin, 100 μg/mL streptomycin and non-essential amino acids (NAA, Gibco) supplemented F12/DMEM (Gibco). Cells were incubated at 37$^{o}$C in a humidified atmosphere (95% air and 5% $CO_2$).

## 2.2.3. Dual-Glo Luciferase reporter gene assay

### *2.2.3.1. Plasmid transfection*

Details of all the plasmids can be found in Section 2.1.3 Plasmids and Viruses. 0.5 μg each of pFR-luciferase and pRL-TKRenilla as well as pRC-APP-Gal4, pRC-APP-Gal4Δ or pRC-APPswe-Gal4 were transfected into primary cortical neurons at 5 DIV in 12 well plates (3.3 x $10^5$ cells per well) using 1 μl/well Lipofectamine 2000 (Invitrogen). The DNA and Lipofectamine were first prepared separately in Opti MEM reduced serum media (75 μL per well) (Invitrogen). These were then combined in 1:1 ratio and incubated at RT for 25 min. 150 μl per well of the DNA/Lipofectamine mix was added to 12 well plates; the neurons were then placed at 37$^{o}$C to allow transfection to take place.

### *2.2.3.2. Treatments and assay*

Treatments were carried out as detailed in Figure legends. 24 h after transfection (unless otherwise stated), the cells were lysed using 100 μl 1x Glo lysis buffer and incubated at RT whilst rocking for 15 min. Firefly luciferase and Renilla luciferase expression was then quantified utilising the Promega Dual Glo luciferase assay kit according to the manufacturer's instructions. Briefly, lysed cells were transferred to a 96 well microplate and 100 μL of 1x Dual Glo substrate was added, the plate was incubated in the dark for 10 min at RT followed by measurement of the first set of readings detected by a luminometer. The plate was then removed and 100 μL of the second Stop and Glo reagent, which quenches the first signal and activates the Renilla luciferase signal was added and incubated in the dark for 10 min at RT and detected by a luminometer. The luciferase signals were measured using a FluorBMG

microplate luminometer. The Firefly signals were normalised as a ratio of the *Renilla* constitutive readings.

## 2.2.4. Site-directed mutagenesis of pRC-APP-Gal4 plasmid

### *2.2.4.1. Primer design*

Mutagenic oligonucleotide primers were designed according to the manufacturer's guidelines. These guidelines included both forward and reverse primers annealing to the same sequence on opposite strands of the plasmid; primers of between 25 and 45 base pairs in length, melting temperature ($T_m$) ≥78$^{o}$C; desired mutation(s) located in the centre of primer sequence with 10-15 bases of correct sequence each side; a minimum GC content of 40% and terminal C or G bases. Two complimentary oligonucleotides were synthesised containing the desired double point mutation, flanked by unmodified nucleotide sequence (see Table 2.3).

### *2.2.4.2. Site direct mutagenesis*

Mutant strand synthesis control and sample reactions were prepared according to manufacturer's instructions (QuikChange II XL site-directed mutagenesis kit, Agilent Technologies, La Jolla, USA) in thin-walled PCR tubes (SLS, Hessle, UK). Each reaction was then placed in the thermocycler and cycled according to the cycling parameters in Table 2.4. Following amplification, the amplification products were digested with 1 µL/reaction Dpn1 restriction enzyme (10U/ µL) added directly to the amplication reaction. The mixture was then briefly centrifuged at maximum speed and immediately incubated at 37$^{o}$C for at least 1h to digest the parental plasmid DNA.

### *2.2.4.3. Transformation of Ultracompetent Cells*

XL10-Gold Ultracompetent cells were gently thawed on ice and 45 µL aliquots transferred to pre-chilled 14 mL BD Falcon polypropylene round bottom tubes. 2 µL of β-mercaptoethanol mix was added to each tube and the contents swirled gently, then incubated on ice for 10 min, swirling every 2 min. TopTen Chemically competent cells were gently thawed on ice (Invitrogen, Paisley, UK). 2 µL of the Dpn 1-treated control and sample reactions were transferred to separate aliquots of competent cells, gently mixed and incubated on ice for 30 min. Competent cell mixtures were then heat shocked for 45 seconds at 42$^{o}$C and transferred back to ice for 2 min. 250 µL of SOC media was added to each transformation tube and incubated at 37$^{o}$C for 1h. 200 µL transformation media was then plated onto LB agar + ampicillin (50 µg/mL) plates and plates were incubated at 37$^{o}$C for more than 16h.

#### *2.2.4.4. Plasmid DNA purification*

QIAprep spin miniprep kits were used according to manufacturer's instructions. Single colonies were picked into 5 mL LB + ampicillin (50 µg/mL) and incubated at 37$^{o}$C on a shaker O/N. The culture was pelleted (6800x g for 3 min at RT) and completely resuspended by pipetting. Cells were lysed and filtered using a QIAprep spin column. Cell supernatant was washed and eluted in RNase free distilled water. DNA quantified by Nanodrop spectrophotometer.

#### *2.2.4.5. Agarose gel electrophoresis*

5x loading buffer was added to purified DNA preparations (final concentration 5% glycerol, 0.042% xylene cyanol, 0.042% bromophenol blue). 0.8 % agarose was heated in TBE buffer (90mM Tris Base pH 8, 90mM Boric acid, 2mM EDTA) and 0.1µl/ml CYBR$^{TM}$ Safe, added before the gel was poured and set. The gel was covered in TBE buffer and DNA preparations were electrophoresed: 20µl of sample was added to each well, and run at 90mV for 45 min. 1 kb DNA ladder, with a separation range of 500 – 10000 kbp (New England BIolabs, Ipswich, USA) was added to enable the calculation of fragment size. Plasmid DNA was visualised by

staining under UV transillumination. The size of the plasmid was determined from the known size of the ladder markers.

#### *2.2.4.6. Restriction enzyme digestion*

1 unit of Not1 (New England Biolabs) was incubated with plasmid DNA (1µg) and 1x NEB buffer 3.1 in a 50 µL reaction made up with endonuclease free water for 60 min at 37$^{o}$C.

#### *2.2.4.7. Plasmid sequencing*

Potential mutant DNA samples were sent for sequencing at Source Bioscience. Samples were provided as 100 ng/µL pure DNA preparations. Primers used to confirm mutagenesis of pRC-APP-Gal4 were: 5'-ACTTACCTCTCAAGTCGGACC-3' (forward primer) and 5'-TCGTAGTGGTTCCACTACTGC-3' (reverse primer). Freeware lalign was utilised to align sequence to template sequence of WT APP.

## 2.2.5. Immunofluorescence

#### *2.2.5.1. Immunostaining*

Primary neurons at 5 DIV (plated at 3.3 x $10^5$ cells per well), cultured on 13 mm glass coverslips (SLS, Hessle, UK) were washed 1x cold PBS and fixed at RT in PBS, pH 7.4, containing 4% paraformaldehyde for 20 min. The cells were then washed with PBS and then incubated in blocking permeabilisation buffer (PBS, pH 7.4 containing 5% skimmed milk powder, 0.1% triton X-100) for at least 15 min. Primary antibodies were applied in antibody buffer (PBS, pH 7.4, containing 1% skimmed milk powder, 0.1% triton X-100) and incubated at RT O/N. The cells were washed 3 times for 5 min in PBS followed by application of Alexa Fluor secondary antibodies in antibody buffer. DAPI was added at 600 nM in PBS (Sigma). Coverslips were mounted in Mowiol and allowed to set at RT O/N. (See Table 2.5 for details).

| Primer name | $T_m$ (°C) | GC content (%) | Primer length | Sequence |
|---|---|---|---|---|
| **APPsweFW** | 79.2 | 44 | 36 | GAGATCTCTGAAGTGAATCTGGATGCAGAATTCCGA |
| **APPsweRev** | 79.2 | 44 | 36 | TCGGAATTCTGCATCCAGATTCACTTCAGAGATCTC |

Table 2.3: **Mutagenesis primers for production of pRC-APPswe-Gal4**

| Segment | Cycles | Temperature (°C) | Time |
|---|---|---|---|
| **1** | 1 | 95 | 1 min |
| **2** | 18 | 95 | 50 sec |
| | | 60 | 50 sec |
| | | 68 | 1 min/kb plasmid* |
| **3** | 1 | 68 | 7 min |

* Control sample extension time was 5 min; reaction sample extension time was 12 min.

Table 2.4: **PCR parameters**

Cycling parameters for side directed mutagenesis of pRC-$APP_{695}$-Gal4

#### *2.2.5.2. Image acquisition*

Stained cells were studied on a Zeiss LSM510 laser-scanning confocal microscope and images taken. LSM image examiner in ImageJ was used to analyse the images.

## 2.2.6. Immunoblotting

#### *2.2.6.1. Sample preparation*

Primary cortical neurons cultured in 6 well plates (6.6 x $10^5$ cells per well) for 6-7 DIV were treated, as detailed in figure legends, with treatments being added directly to the conditioned media. Following treatment, the media was aspirated and wells were washed once with cold PBS. 200 µL of 1x SDS-PAGE Laemmli sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.0025% Bromophenol blue, 1x Complete protease inhibitor cocktail and 1x Complete phosphatase inhibitor cocktail) was added to each well. Wells were then scraped and cellular material transferred to fresh microcentrifuge tubes. These were then centrifuged (2000xg for 5 min) and the supernatant transferred to a new microcentrifuge tube, which was then boiled (100$^o$C for 5 min), cooled and placed in -20$^o$C freezer.

For conditioned media samples, media was collected in separate microcentrifuge tubes, incubated on ice for 10 min, and centrifuged (13,000 rpm for 5 min). 120 µL media sample transferred to a new microcentrifuge tube and 40 µL 1x SDS-PAGE Laemmli sample buffer added. Samples were mixed and placed in -20$^o$C freezer.

| Primary antibody | Source | Details | | Concentration | Secondary antibody | Concentration |
|---|---|---|---|---|---|---|
| **Tau** | Chemicon | Axonal marker | Mouse monoclonal | 1:500 | Goat anti- mouse Alexa Fluor 568 | 1:1000 |
| **MAP2** | Millipore | Dendritic marker | Rabbit polyclonal | 1:1000 | Goat anti-rabbit Alexa Fluor 488 | 1:1000 |

Table 2.5: **Primary and secondary antibodies used for immunofluorescence**

### *2.2.6.2. Tris-Glycine SDSPAGE*

8% polyacrylamide Tris-Glycine resolving gels were prepared (0.375 M Tris-HCl, 0.1% SDS, pH 8.8, 8% acrylamide, 0.5 mg/mL ammonium persulphate (APS), 0.06% TEMED). A clean interface was ensured by overlay of 100% isopropanol whilst the gel set. Resolving gel was overlaid with 4% stacking gel (0.125 M Tris-HCl, 0.1% SDS, pH 6.8, 4% acrylamide, 0.5 mg/mL APS, 0.06% TEMED). Tris-Glycine gels were run in 1x Tris-Glycine SDS-PAGE running buffer (0.025M Tris, 0.192 M glycine, 0.1% SDS, pH 8.6, National Diagnostics). Prepared samples were loaded into the gel with a molecular weight marker (SeeBlue Plus marker, Invitrogen) adjacent and run at 90V until bands exit the stacking gel and then 120-130V for approximately 1h in a Bio-Rad mini-PROTEAN Tetra cell.

### *2.2.6.3. Western Blotting*

Filter papers were pre-soaked in blotting buffer (0.025M Tris, 0.192 M glycine, 0.1% SDS, pH 8.6, 20% methanol). Nitrocellulose membranes (0.45 µm, GE Healthcare) were pre-soaked in distilled water. Following completion of the electrophoresis, gels removed from cassette and stacking gel removed.

Semidry transfer: Gels were stacked with 4 filter papers, nitrocellulose membrane, gel section and 4 filter papers onto the blotting surface. Proteins were transferred from the gel to the membrane using constant amperes (1.5 $mA/cm^2$) for 1h.

Wet transfer: Gel were equilibrated in blotting buffer then stacked into blotting cassette with sponge, 2 filters, gel, membrane, 2 filters, and sponge. The cassette was then placed in the blotting apparatus and filled with blotting buffer. Proteins were transferred from the gel onto the membrane using constant amperes (0.4A for 1h 05).

Following completion of transfer, membranes were washed 1 x 5 min TBS-T (0.02 M Tris, pH 7.5, 0.1% Tween-20, 0.150 M NaCl). Membranes were then blocked in 5% milk blocking solution (5% milk powder in TBS-T) for 30 min after which membranes

were washed 1x 5 min TBS-T and incubated with primary antibody in antibody buffer (1% milk powder in TBS-T) O/N at 4$^{o}$C on a rocker (See Table 2.6 for details).

#### *2.2.6.4. Protein detection*

Following appropriate primary antibody incubation, membranes were washed 2 x 5 min in TBS-T. Complimentary secondary antibodies conjugated to horseradish peroxidase (HRP) were then applied in antibody buffer for 45 min at RT on a rocker. (See Table 2.6 for details). Membranes were then washed 2 x 5 min with TBS-T and 1 x 5 min with TBS (0.02 M Tris, pH 7.5, 0.150 M NaCl). Following the final wash, excess fluid on the membrane was blotted away and the membrane placed flat on a clean surface. The required volume of ECL detection fluid was made up and applied to the membrane for 1 min. Excess fluid was once again blotted away and the membrane placed in a Fusion SL Chemiluminescence system to be visualised.

Membranes from the wet method of western blotting were placed in an autoradiography film cassette and hyperfilm autoradiography films (GE Healthcare) were exposed to the membranes in the dark for varying lengths of time. They were then placed in a Fuji LAS 4000 X-ray processor to be developed.

## 2.2.7. Mouse A$\beta_{1\text{-}40}$ ELISA

Colorimetric BetaMark x-40 Aβ ELISA was performed according to manufacturer's instructions (Covance, Princeton, USA). Prepared standard samples (100 µL) were prepared and applied to the 96 well capture antibody coated plate in duplicate. Experimental samples diluted 1:2 with dilution buffer (100 µL) were applied in triplicate, covered and incubated O/N at 4$^{o}$C. The next day, the plate was washed five times with wash buffer and then 200 µL/well substrate was applied and incubated for 45 min in the dark. The luminescence levels were then read at 620 nm.

| Primary antibody | Source | Details | | Concentration | Secondary antibody | Concentration |
|---|---|---|---|---|---|---|
| **p44/42 p-MAPK** | Cell signalling | pThr202/p-Tyr204 (pERK) | Rabbit polyclonal | 1:2000 | Goat-anti-rabbit HRP | 1:5000 |
| **ERK2** | Santa Cruz Biotechnology | C-14 | Rabbit polyclonal | 1:5000 | Goat-anti-rabbit HRP | 1:5000 |
| **ADAM10** | Millipore | 735-749 | Rabbit polyclonal | 1:2000 | Goat-anti-rabbit HRP | 1:5000 |
| **BACE1** | Sigma | 2C13 | Mouse monoclonal | 1:2000 | Goat-anti-mouse HRP | 1:5000 |
| **APP** | Millipore | 22C11 | Rabbit polyclonal | 1:5000 | Goat-anti-rabbit HRP | 1:5000 |
| **sAPPα** | Kind gift of SFL | 5G11 specific for murine sAPPα | Rat monoclonal supernatant | 1:9 | Goat-anti-rat HRP | 1:5000 |
| **sAPPα** | Kind gift of SFL | 14D6 specific for human sAPPα | Rat monoclonal supernatant | 1:9 | Goat-anti-rat HRP | 1:5000 |
| **sAPPβ** | Kind gift of SFL | 192WT | Rabbit polyclonal | 1:100 | Goat-anti-rabbit HRP | 1:5000 |
| **β-tubulin** | Sigma | Anti Tuj1 441-450 amino acids of β-III tubulin | Mouse monoclonal | 1:25,000 | Goat-anti-mouse HRP | 1:5000 |
| **β actin** | Sigma | AC-74 | Mouse monoclonal | 1:5000 | Goat-anti-mouse HRP | 1:5000 |

Table 2.6: **Primary and secondary antibodies used for immunoblotting**

## 2.2.8. BACE1 activity detection

The cell free BACE1 activity assay is based on fluorescence resonance energy transfer (FRET) in which a fluorescent signal is observed following substrate cleavage by BACE1. The BACE1 activity detection assay was performed according to manufacturer's instructions (Sigma, Missouri, USA). Assay components were mixed to a final volume of 100 µL in a black 96 well microplate. Test samples were incubated with inhibitors or flavonoid compounds as indicated in figure legends. Fluorescence readings (excitation 320 nm, emission 405 nm) were taken at time zero and then incubated at 37$^{o}$C for 2h and the fluorescence levels measured again using a Molecular Devices VERSAmax microplate reader. Stop solution was then added to stabilise the signal for 24h.

In order to measure endogenous BACE1 activity levels in primary cortical cultures in 12 well plates 3.3 x $10^5$ cells per well) for 6 DIV were treated, as detailed in figure legends, with treatments being added directly to the conditioned media. Following treatment, the media was aspirated and wells were washed once with cold PBS. 200 µL of CelLytic M (Sigma, Missouri, USA) was added to each well. Wells were then scraped and cellular material transferred to fresh microcentrifuge tubes. These were then centrifuged (2000xg for 5 min) and the supernatant transferred to a new microcentrifuge tube. 30 µL cell lysates were then incubated with synthetic BACE1 substrate and fluorescent levels measured as per the cell free assay protocol.

## 2.2.9. Phase contrast microscopy

Morphological assessment of neuronal viability and phenotype was made by phase contrast microscopy. Images were captured with a Nikon E5000 digital camera using a NikonEclipse TS100 inverted microscope.

## 2.2.10. Viral transduction

Details of pAd5-Egr1 can be found in Section 2.1.3: Plasmids and viruses and Appendix 3. $1x10^5$ plaque forming units (pfu)/mL Ad5-Egr1-luciferase was transduced into primary cortical neurons at 6 DIV in 24 well plates ($6.6x10^5$ cells per well). Briefly, conditioned media was removed from the neurons and ½ volume fresh media containing viral dilution was added to the cells and incubated at 37°C for 4h. Media was then removed and 1x volume original conditioned media replaced onto the neurons. Neurons were then treated as detailed in figure legends. 24h post transduction, media was removed from the cells and 50 µL 1x Glo lysis buffer was added and incubated at RT whilst rocking for 15 min. Lysed cells were transferred to a white 96 well plate and 50 µL 1x Dual Glo substrate was added, the plate was incubated in the dark for 10 min at RT followed by measurement using a FluorBMG microplate luminometer.

## 2.2.11. Quantification and Statistics

Immunoblot ECL bands captured using the Fusion SL Chemiluminescence system were quantified using FUSION-CAPT analysis software (Peqlab). Immunoblot Hyperfilm ECL bands were quantified by scanning into NIH Image J software at a resolution of 1200 dots per inch (dpi) using an Epson Perfection V700 Photo flatbed scanner, and the mean background corrected optical density (OD) of each band was interpolated from an OD calibration curve created using an OD step tablet. Mean data ± S.E.M. were graphed using Graph Pad Prism 6 software. APP-Gal4 luciferase assay, immunoblot, viral transduction and Aβ ELISA data were analysed by one-way ANOVA with Bonferroni post-test, two-way ANOVA with Bonferroni post-test or student's t-test, using Graph Pad Instat software. For the APP-Gal4 luciferase assay the minimal experimental unit (n=1) was defined as a single independent transfection. Differences between treatments and conditions were considered to be statistically significant when $p<0.05$.

# Chapter 3

## 3. Characterisation and validation of an APP-Gal4 reporter assay in primary neurons

# 3.1 Introduction

## 3.1.1 Use of primary cortical cultures to study APP processing

Much of what we currently understand about APP and its metabolism has come from extensive characterisation studies in overexpressing cell lines. Whilst these studies have provided critical new insight into the underlying biochemistry of APP processing, they are much more limited for studying the physiology of APP and the modulation of APP proteolytic pathways. This is chiefly down to a lack of key phenotypic features of mature mammalian neuronal networks. This includes neuronal specific cellular architecture such as synapses, associated specialised protein expression such as at the post synaptic density (PSD) and spontaneous action potentials. Primary neurons have provided a crucial model in which detailed elucidation of APP processing regulation has been performed. Due to their faithful representation of neurons *in situ*, expressing the full complement of synaptic proteins and containing all neuronal specific cellular compartments, they are currently the best model for studying physiological regulation of APP processing.

## 3.1.2 Methods for functional readout of APP processing in primary neurons

In this chapter, the characterisation and development of a cell based reporter gene assay in primary cortical neurons has been described. Transfection of a plasmid expressing full length $APP_{695}$ conjugated to the yeast transcriptional factor Gal4 into primary cortical neurons and subsequent measurement of Gal4-dependent luciferase reporter gene expression, allowed analysis of changes in $\gamma$-secretase mediated APP processing. APP-Gal4 assays have been used successfully in previous

studies to investigate APP processing-dependent activities. Two groups have described different utilities for an APP-Gal4 plasmid (Cao and Sudhof, 2001, Gianni et al., 2003). An APP-Gal4 construct was first used to investigate the ability of APP to activate gene expression. Full length $APP_{695}$ was fused with Gal4 either at the cytoplasmic boundary of the TMD of APP or at the N terminus of the protein. It was through this study that the tripartite complex of APP, Fe65 and Tip60 was shown to be transcriptionally active (Cao and Sudhof, 2001). The second study utilised a different fusion protein consisting of human $APP_{695}$ at the N terminus and the yeast transcription factor Gal4 joined by six glycine residues at the C terminus (APP-Gal4). This was used to identify extracellular signals that induced γ-secretase mediated processing. The APP-Gal4 plasmid was used in combination with a chloramphenicol acetyltransferase (CAT) reporter plasmid to visualise modulation of APP processing (Gianni et al., 2003) and identified PDGF as an activator of amyloidogenic processing, providing some of the earliest evidence for tyrosine kinase receptor regulation of APP processing.

The reporter assay described in this thesis, which utilises the same APP-Gal4 fusion protein as Gianni et al (2003), provides a functional readout of APP processing. This is not possible through alternative methods of APP measurement such as biochemical analysis of APP carboxy-terminal fragments (CTFs) or sandwich ELISA assays of Aβ secretion. Analysis of APP CTFs (the membrane tethered products of α- and β-secretase cleavage), by immunoblot is a powerful technique that allows detailed investigation of intracellular secretase mediated changes in APP processing. However, this technique is technically challenging and low throughput, therefore unsuitable for screening studies such as that proposed for this study. Measurement of pathway-specific, soluble APP ectodomain and Aβ secretion in primary neurons, although possible through much simpler methods than CTF analysis, such as specific ELISA assays or standard immunoblotting, present with different technical problems. Analysis of changes in these products is complex due to the significant levels of these stable products in the culture medium as a result of constitutive APP processing during neuronal culture maturation. Through simple manipulation of culture conditions or the APP-Gal4 gene construct, this system has

the potential to allow regulatory effects of APP mutations, post-translational APP modifications or general cell metabolism manipulations, on secretase-mediated APP processing to be analysed.

In order to establish whether this assay could be utilised in primary neurons to identify novel modulators of APP processing, it was subject to validation and known environmental and genetic perturbations associated with AD. These challenges will determine whether the assay is sensitive to known modulators of APP processing and therefore whether it can be used to screen for novel flavonoid modulators of γ-secretase mediated APP processing.

# 3.2 Results

## 3.2.1 Characterisation of neuronal model systems

This thesis of work has been carried out almost entirely using mouse embryonic cortical cultures. Neuronal cells are terminally differentiated, post mitotic cells that have exited the cell cycle. This property of neurons has made the development of high quality neuronal cell lines, which accurately recapitulate the physiology and cell biology of neurons extremely difficult and none exist that come close to modelling the complexity of a functional mammalian synapse. Therefore, the use of primary cell cultures, although more labour intensive and technically challenging to prepare and manipulate are a more faithful *in vitro* model for studying neuronal function in the mammalian brain.

To confirm that the primary cultures prepared according to methods described in Section 2.2.1 had a predominantly neuronal phenotype, morphological analysis of neuronal maturation and cellular staining of neuronal-specific structures were performed. This process was necessary so that results extrapolated from these cultures could be attributed to neuronal responses rather than other neural cell types.

Mouse embryonic primary cortical cultures were stained using anti-MAP2 and anti-tau antibodies in order to identify axons (tau) and dendrites (MAP2). The cultures were also counter-stained with the nucleus marker DAPI. Cells showed extensive dendritic extensions and well-defined axons with numerous potential synaptic connections along the axonal projections, consistent with neuronal morphology (Figure 3.1). Staining for GFAP showed less than 2% GFAP positive cells in these cultures (data not shown).

Phase contrast images taken of the neurons as they matured in culture initially showed highly polarised cells with large cell bodies (Figure 3.2(A)). As the neuronal cultures developed *in vitro*, the number of fine processes increased, consistent with

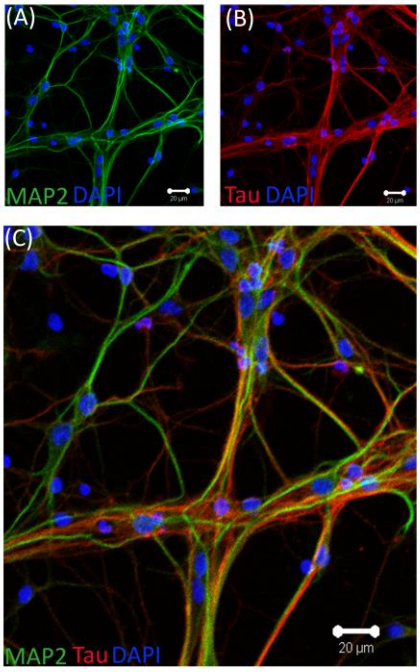



Figure 3.1: **Primary cultures express neuronal markers**

(A-C) Double immunofluorescence staining of primary cultured cortical neurons at 5DIV. Neurons were fixed and double immunostained for MAP2 and tau. (C) Image overlay showing complex network of tau-positive axons and MAP2-positive dendrites. Tau was labelled with mouse monoclonal anti-tau (1:500) (Chemicon) and MAP2 was labelled with rabbit polyclonal anti-MAP2 (1:1000) (Millipore). Nuclei were counterstained with DAPI (blue), scale bars 20 µM.

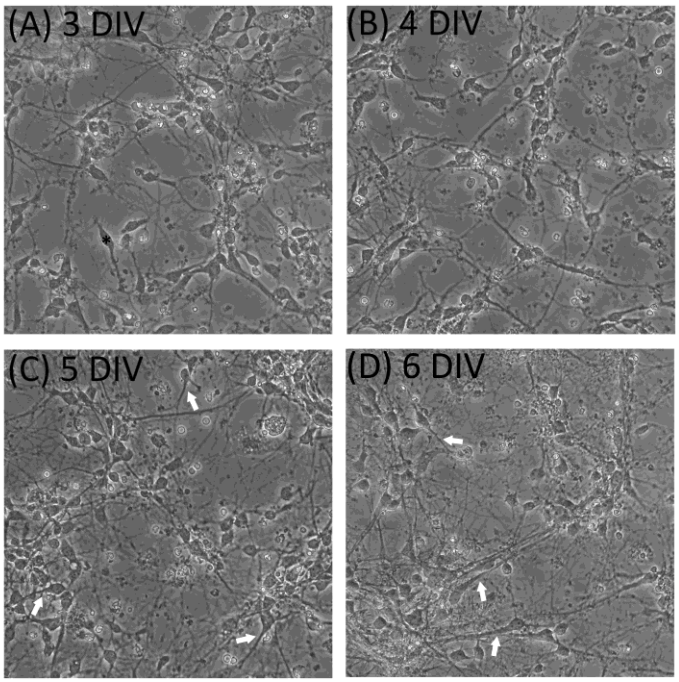



Figure 3.2: **Primary cortical cultures develop extensive network of connections by 5 DIV**

(A-D) Phase contrast images of primary cultured cortical neurons as they develop from 3 DIV-6 DIV. (A) At 3DIV, have polarised cells with large cell bodies (*), as maturation continues, the number of processes increase (B and C) until at 5 DIV there are large, tapering axons (white arrows) with extensive dendritic networks which are maintained at 6 DIV (D)

the maturation of the culture and formation of functional synapses. Supporting the immunocytochemistry results, by 5 DIV single, large, tapering axons extending from the cell bodies can be identified along with extensive, finer dendritic networks confirming these cultures have a neuronal phenotype (Figure 3.2C (white arrows)).

### 3.2.2 An APP-Gal4 gene reporter assay in primary mouse embryonic cultures

Despite the many advantages of primary neurons, the major disadvantage is the technical difficulty of genetic manipulation in these cultures. Despite this, an APP-Gal4 gene reporter assay has previously been described in primary neurons, which allowed measurement of changes in APP processing in response to exogenous stimuli (Hoey et al., 2009). In order to utilise this approach for the identification of modulators of processing, the reporter first had to be thoroughly characterised and revalidated because the laboratory was now using a different mouse strain, CD1 as opposed to C57/BL6 and NIH Swiss White as previously reported.

The APP-Gal4 assay utilises the well-known Gal4-UAS yeast system and takes advantage of the fact that APP is a type 1 transmembrane protein, meaning the holoprotein is retained at cell membranes. The assay measures levels of transfected human $APP_{695}$ processing in primary neurons by coupling this processing, via the Gal4-UAS system, to transcription of a luciferase gene. To enable this to occur, a number of plasmids must be chemically transfected into the primary neurons. The plasmid pRC-APP-Gal4, encodes full length, human $APP_{695}$ with the yeast transcription factor Gal4 inserted in frame at its C terminus via a glycine linker, allowing expression of the fusion protein APP-Gal4 (Perkinton et al., 2004). Following $\gamma$-secretase mediated APP cleavage, an AICD-Gal4 fragment is released, which, unlike the full length protein, is free to translocate and bind to the synthetic, Gal4 specific, UAS promoter of a second plasmid pFR-luciferase, which encodes the Firefly luciferase gene. Firefly luciferase expression can then be quantified utilising a standard luminescence assay as described in Section 2.2.3. A third plasmid, pRL-

TKRenilla is also transfected, which constitutively expresses the Renilla luciferase gene, acting as an internal control to which the Firefly luminescence values can be standardised (Figure 3.3).

### 3.2.3 APP-Gal4 gene reporter assay signal is dependent on Gal4-UAS coupling

Initial work investigated the dependence of the reporter assay luminescent signal on the coupling of the Gal4 yeast transcription factor to the UAS-containing, synthetic promoter of the Firefly luciferase plasmid. It was important to determine that the signal detected was as a result of coupling of the APP-Gal4 cleavage product, AICD-Gal4, to the synthetic promoter of pFR-luciferase and consequent Firefly luciferase expression.

To confirm this, a mutated version of the pRC-APP-Gal4 plasmid and combinations of the required plasmids were transfected and assayed for resultant luciferase expression. The mutated version of APP-Gal4 (pRC-APP-Gal4$\Delta$) contained a single base substitution, introducing a stop codon within the yeast Gal4 gene. This truncated the protein within its DNA binding domain so that it could not activate Firefly luciferase transcription (Hoey et al., 2009).

Transfection of primary neurons with pRC-APP-Gal4$\Delta$ in combination with the pFR-luciferase and pRL-TKRenilla plasmids showed a significant decrease (42%) in luciferase expression compared to transfection with pRC-APP-Gal4 (Figure 3.4). Co-transfection of the neurons with pRC-APP-Gal4 and the control plasmid pRL-TKRenilla showed a 66% decrease in expression compared to pRC-APP-Gal4 with pFR-luciferase and pRL-TKRenilla (Figure 3.4). Transfection carried out with pFR-luciferase and pRL-TKRenilla in combination and pRL-TKRenilla on its own showed minimal luciferase expression, more than 10 times lower than transfection with pRC-APP-Gal4, pFR-luciferase and pRL-TKRenilla (Figure 3.4). This confirmed that the majority of luciferase expression was dependent on coupling of the APP-Gal4 to the promoter of pFR-luciferase.

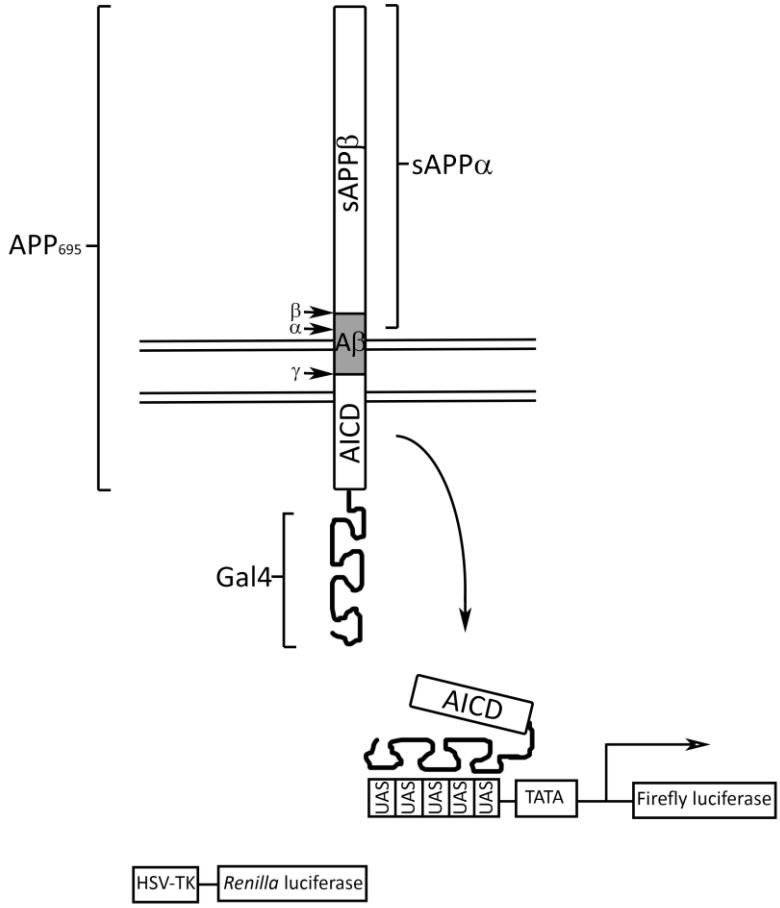



Figure 3.3: **Schematic representation of APP-Gal4 gene reporter assay for measurement of γ-secretase mediated APP processing**

Cleavage by γ-secretase of human $APP_{695}$ fused to the yeast transcription factor Gal4 (APP-Gal4) allows release of AICD-Gal4. Free AICD-Gal4 then binds to the synthetic promoter of Firefly luciferase plasmid consisting of 5 UAS repeats and a TATA box, driving expression of reporter gene Firefly luciferase. Resultant Firefly luciferase expression changes are measured relative to an independent *Renilla* luciferase signal.

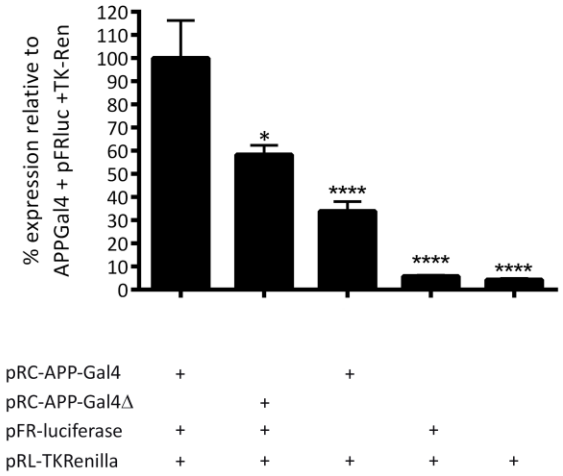



Figure 3.4: **Luciferase expression is dependent on transfection of functional APP-Gal4**

5DIV primary cultured cortical neurons were transfected with pFR-luciferase Firefly luciferase reporter gene plasmid alone and in combination with pRC-APP-Gal4 or pRC-APP-Gal4Δ plasmids encoding for $APP_{695}$-Gal4 and $APP_{695}$Gal4 with a non-functional DNA binding domain. All cells were co-transfected with pRL-TKRenilla that constitutively expresses *Renilla* luciferase. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression. Each column is the mean ± S.E.M. of 6 separate transfections (n=6; * $p<0.05$, ****$p<0.0001$, one way ANOVA with Bonferroni post-test).

### 3.2.4 APP-Gal4 gene reporter assay preferentially reporters βγ-mediated APP processing

Following confirmation of Gal4-UAS dependence of the assay, the next step was to investigate the dependence of the reporter assay on secretase-mediated APP cleavage. This was achieved using commercial inhibitors of the secretase enzymes responsible for APP cleavage.

Primary neurons were treated with the γ secretase inhibitor DAPT, transfected 30 min later and luciferase expression measured 24h post-transfection. DAPT treatment reduced mean luciferase expression by 74% (Figure 3.5) indicating the majority of luciferase expression is driven by γ-secretase-mediated APP cleavage. APP undergoes RIP, therefore initial cleavage by α- or β-secretase activities must occur prior to γ-secretase cleavage (Lichtenthaler et al., 2011). As it has been proposed that the amyloidogenic and non-amyloidogenic pathways exist in equilibrium (Colombo et al., 2012, Suh et al., 2013) and primary rodent neurons appear to favour a BACE1 processing route (Colombo et al., 2012, Hoey et al., 2013), it was necessary to determine the relative contributions of the α- and β-secretase enzyme activities to the luciferase signal. Treatment of transfected neurons with two structurally distinct BACE1 inhibitors, BsI (peptide inhibitor) and BiV (cell-permeable catalytic BACE1 inhibitor), for 24h caused 50% and 38% reductions in luciferase expression respectively (Figure 3.5). Conversely, treatment of neurons for 24h with TAPI, a broad-spectrum metalloprotease inhibitor that reduces α-secretase activity caused a modest 10% increase in luciferase expression (Figure 3.5), suggesting the assay predominantly measured BACE1-mediated, amyloidogenic APP processing.

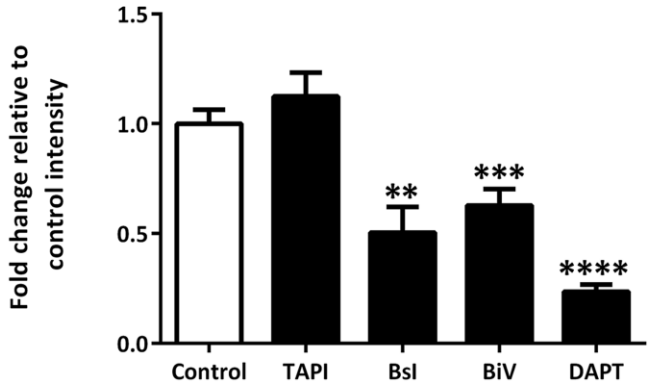



Figure 3.5: **APP-Gal4 reporter gene assay preferentially reports βγ-secretase mediated APP processing in primary cortical neurons**

5DIV primary cultured cortical neurons were treated with vehicle (Control), 50 µM TAPI, 10 µM BsI, 10 µM BiV, or 10 µM DAPT for 30 minutes prior to co-transfection with pRC-APP-Gal4, pFR-luciferase and pRL-TKRenilla plasmids. Inhibitors were present for throughout the experiment. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression. Each column is the mean ± S.E.M. of 12-18 separate transfections (n=12-18; ** $p<0.01$, *** $p<0.001$ ****$p<0.0001$, one way ANOVA with Bonferroni post-test).

### 3.2.5 Fe65 enhances reporter-dependent luciferase expression in mouse primary cultured neurons

The APP-Gal4 gene reporter assay has been confirmed as dependent upon the AICD conjugated to the Gal4 transcription factor binding to the synthetic promoter of the pFR-luciferase plasmid. Due to the poor transfection efficiency of primary neurons, the low level of luciferase expression made changes between basal and modulated conditions difficult to detect. In order to increase the luciferase signal, allowing greater disparity between basal and APP processing-dependent luminescence, a physiological stabiliser of AICD, the adaptor protein Fe65 was employed. Via its YENTPY motif in the PTB2 domain, Fe65 binds directly to the AICD (Fiore et al., 1995), stabilising AICD in the cytoplasm prior to nuclear translocation (Kimberly et al., 2001). Transfection with Fe65 was thought to be an effective way to increase the luciferase signal through stabilization of the AICD-Gal4 product.

To determine the influence of Fe65 on luciferase expression, pC1-CMV-Fe65 was co-transfected with the required assay plasmids and luciferase expression was measured 24h later. This enhanced luciferase expression ~10 fold (Figure 3.6), confirming that the assay was sensitive to physiological stabilisers of the AICD and allowing greater disparity between basal and induced luciferase expression.

### 3.2.6 APP-Gal4 reporter gene assay is sensitive to pathological challenges

To further the potential utility of the assay, its sensitivity to known pathological cellular challenges associated with AD was investigated. To probe the sensitivity of the assay to AD-relevant cellular insults, differing paradigms were developed. The assay was carried out following neuronal maturation in antioxidant-free conditions and following transfection of a mutated version of the pRC-APP-Gal4 to introduce a known FAD-causing mutation.

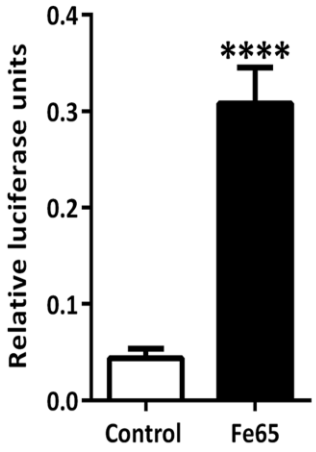



Figure 3.6: **Fe65 significantly enhances APP-Gal4 reporter gene expression**

5DIV primary cultured cortical neurons were co-transfected with pRC-APP-Gal4, pFR-luciferase and pRL-TKRenilla plasmids alone and also with the addition of pC1-CMVFe65 plasmid. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression. Each column is the mean ± S.E.M. of 18 independent transfections across 3 biological replicates (n=3; ****$p<0.0001$, Student's t-test).

### *3.2.6.1 Neuronal maturation in antioxidant free media enhances reporter dependent luciferase expression*

In the AD brain, the cellular environment changes and cells are subjected to greater levels of oxidative stress, lower glucose levels and higher concentrations of protein aggregates (Markesbery, 1997). These conditions are thought to be some of the main environmental factors that contribute to enhanced pro-amyloidogenic APP processing, as oxidative stress is seen prior to increases in Aβ concentration (Smith et al., 2000).

To test the sensitivity of the reporter assay to a pro-oxidant environment, a stress model had to be established in primary neurons. Antioxidants are essential for neuronal survival *in vitro,* however the window of necessity is within the first 24h post explantation only, after which antioxidants can be removed with little resultant cell death (Perry et al., 2004). This allowed a model of oxidative stress to be tested where the oxidative stress is generated by chronic removal of endogenous antioxidants rather than addition of exogenous oxidants.

In primary cell culture, the major source of antioxidants is the B27 media supplement. This supplement contains reduced glutathione, tocopherol (vitamin E), catalase and SOD (Perry et al., 2004). To determine the effect of antioxidant withdrawal on APP cleavage-dependent luciferase expression, culture media was changed on 1 DIV to Neurobasal lacking antioxidants (B27 AO-free) or Neurobasal containing antioxidants (B27 AO+) and cells were maintained under physiological conditions.

Phase contrast images were taken on 3 and 5 DIV to assess morphological changes occurring in B27 AO-free cultures compared to B27 AO+ cultures. These showed that the antioxidant free cultures had a slightly higher incidence of apoptotic cells, appearing as small, compact floating cells, however there was no widespread death (Figure 3.7(Aiii and iv)). Cell bodies appeared to be unaffected; however the dendritic networks developed in the antioxidant free media were much less

extensive compared to those that developed in the presence of antioxidants (Figure 3.7(A)). The microscopic analysis therefore suggests that any changes seen in the APP-Gal4 gene reporter assay were not due to gross morphological changes or widespread cell death. Neurons that had matured in both the presence and absence of antioxidants were transfected and levels of luciferase expression measured 24h later. APP cleavage dependent luciferase expression more than doubled when compared to neurons cultured in the presence of antioxidants (Figure 3.7(B)). This lends support to the hypotheses that oxidative conditions drive amyloidogenic APP processing or increase APP expression.

#### *3.2.6.2 Introduction of the Swedish mutation in APP enhances reporter-dependent luciferase expression.*

BACE1 sensitivity of the assay predicted that familial AD mutations known to enhance Aβ production, such as the Swedish mutation of APP (K670N/M671L) (Citron et al., 1992), would increase luciferase expression. To investigate the sensitivity of the APP-Gal4 reporter to known disease-causing mutations in APP and to generate a cellular model of pathogenic APP processing, site-directed mutagenesis of pRC-APP-Gal4 was undertaken. A double point mutation causing two amino acid substitutions, lysine to arginine and methionine to leucine (K670N/M671L), was introduced.

Initially, PCR amplification using mismatched primers that introduced the double point mutation was carried out (Figure 3.8(A)) (see Section 2.2.4). Transformed clones were identified by DNA electrophoresis (Figure 3.8(B)). To ensure no gross DNA changes had occurred during the mutagenesis, a unique restriction site (Not1) digest was performed to linearise the plasmid DNA and assess total plasmid size of all mutants. Undigested pRC-APP-Gal4, digested pRC-APP-Gal4 and digestions of the 3 putative pRC-APPswe-Gal4 mutants were compared. These showed there were no gross changes in size in the putative mutant plasmids (Figure 3.8(c)). The 3 mutants were therefore sent for sequencing; alignment with the known WT $APP_{695}$

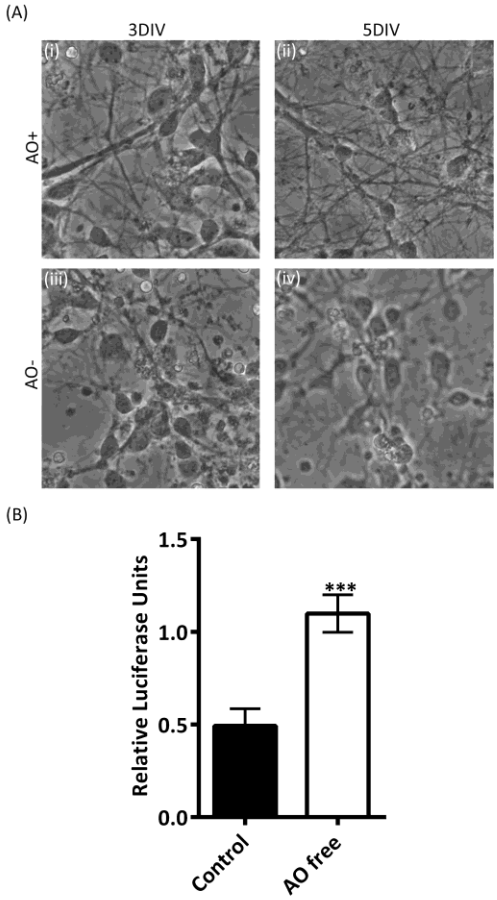



Figure 3.7: **Antioxidant withdrawal enhances βγ-mediated APP processing in primary neurons in the absence of gross neuronal cell loss**

(A) Phase contrast images of neuronal cell development at 3 DIV and 5 DIV in (i and ii) the presence of antioxidants and (iii and iv) in the absence of antioxidants. Primary cultured neurons were prepared and plated out in Neurobasal +B27 media. At 1 DIV, media was removed and replace with Neurobasal +B27 (+AO) or Neurobasal +B27 minus antioxidants (-AO). Cultures were then allowed to mature to 5DIV. Phase contrast images were taken with 400x magnification. (B) 5DIV primary cultured cortical neurons matured in the presence (Control) or absence (AO free) of antioxidants were co-transfected with pRC-APP-Gal4, pFR-luciferase and pRL-TKRenilla plasmids. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression. Each column is the mean ± S.E.M. of 18 independent transfections across 3 biological replicates (n=3; ***$p<0.001$, Student's t-test).

sequence confirmed that pRC-APPswe-Gal4 #3 contained the double point mutation leading to K595N/M596L amino acid substitutions (Figure 3.8(D)).

Following successful identification of the mutant, the level of luciferase expression resulting from pRC-APPswe-Gal4 compared to pRC-APP-Gal4 was investigated. Primary neurons were transfected with either pRC-APP-Gal4 or pRC-APPswe-Gal4 in the presence and absence of the γ-secretase inhibitor DAPT. Luciferase expression was measured 24h later. Transfection with pRC-APPswe-Gal4 resulted in a 2-fold increase in luciferase expression compared to non-mutated APP, whilst remaining sensitive to DAPT (Figure 3.9).

Collectively this chapter characterised the assay as being dependent on Gal4-UAS coupling and γ-secretase mediated APP cleavage. Taken together, the Fe65, antioxidant withdrawal and Swedish mutation results confirmed the assay tractability and the resultant activity shifts were consistent with the assay being predominantly a reporter of βγ-secretase-mediated amyloidogenic APP processing.

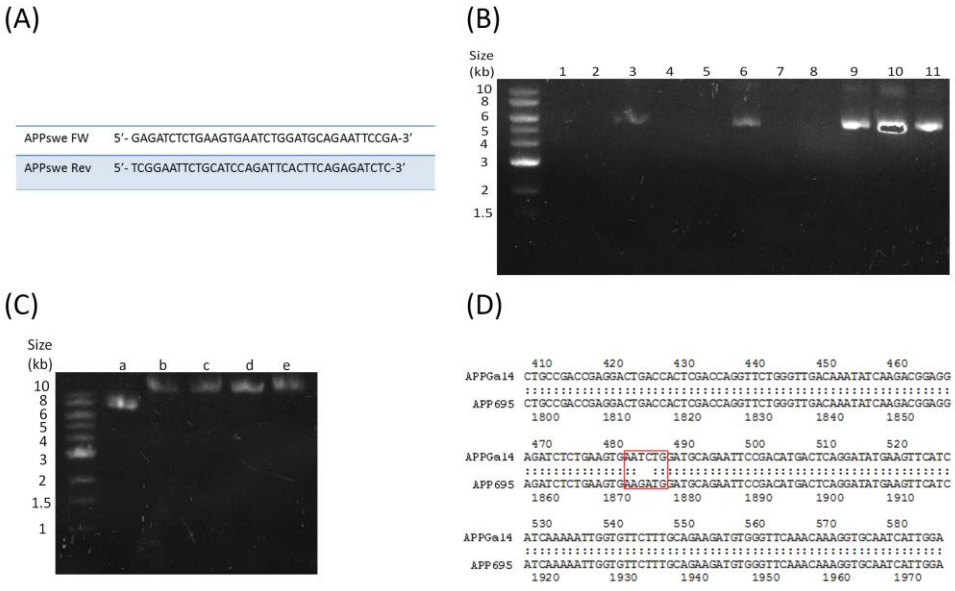



Figure 3.8: **Design and production of APPswe-Gal4 plasmid**

(A) Mutagenesis primers designed to introduce double point mutation into APP sequence of pRC-APP-Gal4. (B) Identification of plasmid DNA-containing miniprep samples. 0.8% agarose gel was run for 30 min of 11 samples loaded with 6x DNA loading buffer: 1-8 – samples transformed using XL Gold competent cells, single ampicillin resistant colonies were picked into 5mL LB broth and cultured O/N followed by standard DNA extraction and purification; 9-11 – samples transformed using TopTen chemically competent cells, single ampicillin resistant colonies were picked into LB broth and cultured O/N followed by standard DNA extraction and purification. (C) Three TopTen putative APPswe-Gal4 (c-e) and original APP-Gal4 (b) plasmid DNA preparations were digested with Not1 for 4h at 37$^{o}$C to linearise DNA. Samples were run on 0.8% agarose gel alongside undigested control plasmid (a) for 30 min. (D) Sequence alignment of clone #3 APPswe-Gal4 sequence with $APP_{695}$ cDNA sequence. Red box highlighting double point mutation.

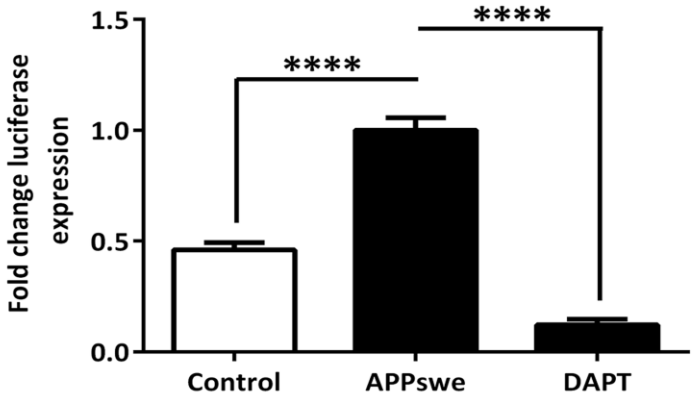



Figure 3.9: **Swedish APP mutation enhances βγ-mediated APP processing in primary neurons in a DAPT dependent manner**

5 DIV primary cultured cortical neurons were co-transfected with APP-Gal4 plasmid point mutated at 'Swedish' site (K670N/M671L; APPswe) or WT APP-Gal4 (Control), with pFR-luciferase and pRL-TKRenilla. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression. Co-transfection with APPswe-Gal4 caused increase in luciferase expression compared to WT that was blocked by DAPT treatment (10 µM for 24h) Each column is the mean ± S.E.M. of 24 independent transfections across 4 biological replicates (n=24 ****p<0.0001, One way ANOVA with Bonferroni post-test).

## 3.3 Discussion

In this chapter the APP-Gal4 gene reporter assay is confirmed as a sensitive method for assessment of APP processing modulation in primary neurons. Utilising commercial inhibitors of α- , β- and γ-secretase, it has been shown that the assay is strongly dependent on γ-secretase processing and preferentially reports βγ-secretase-mediated processing. This is in agreement with previous reports utilising this assay in primary neurons (Hoey et al., 2009). The assay was stimulated by co-expression of Fe65, introduction of APP mutations known to favour Aβ formation and following antioxidant withdrawal from the media.

### 3.3.1 The APP-Gal4 reporter assay preferentially measures changes in amyloidogenic APP processing

The APP-Gal4 reporter assay was sensitive to two, structurally unrelated, commercial β-secretase inhibitors (BsI and BiV) and a commonly used γ secretase inhibitor (DAPT). In contrast, the assay was not inhibited by a broad spectrum metalloprotease inhibitor (TAPI), known to inhibit α-secretase activity. Due to the complexity of APP processing regulation in primary neurons, there are a number of possibilities to explain why the assay preferentially reported βγ-mediated APP processing. The most basic explanation is that primary neurons do not undergo processing via the non-amyloidogenic pathway. However, despite neurons being unusual in exhibiting a higher proportion of amyloidogenic compared to non-amyloidogenic processing, proteolytic fragments of the non-amyloidogenic pathway are easily detectable by western analysis and endogenous APP shows sensitivity to TAPI in primary neurons (Hoey et al., 2009, Colombo et al., 2012).

Physiological control of APP processing is heavily reliant on spatial segregation of APP and secretase enzymes and therefore an alternative explanation is the

accumulation of APP-Gal4 in cellular locations associated with high BACE1 activity such as endosomes. Due to the low transfection efficiencies of the neurons, it was not possible to investigate this possibility further, however as spatial segregation plays a crucial role in APP processing regulation this is an attractive hypothesis (Haass et al., 2012).

Alternatively, apparent preference for βγ mediated processing could be due to different activities of the AICD-Gal4 processing product from the amyloidogenic and non-amyloidogenic pathways. It may be that only the AICD-Gal4 fragments released from βγ-mediated processing have the ability to transactivate gene expression of the target reporter plasmid. The AICD has been shown to transactivate gene expression via formation of a complex involving adaptor protein Fe65 and histone acetyltransferase Tip60 (Cao and Sudhof, 2001). It has been suggested that only AICD produced via amyloidogenic processing is transcriptionally active and therefore responsible for nuclear signaling (Goodger et al., 2009, Belyaev et al., 2010). This assay is a measure of secretase mediated processing, not a measure of the transcriptional activation activity of AICD, as it is the Gal4-UAS coupling that leads to activation of the luciferase gene, however evidence suggesting only AICD from the amyloidogenic pathway is able to form functional units with adaptor proteins provides a rationale to explain the βγ-mediated preference of the APP-Gal4 assay. Studies of AICD transcriptional activity in primary neurons however, have not established a difference in functionality of AICD generated from the amyloidogenic or non-amyloidogenic pathways and so this hypothesis remains unproven.

Differences in results from studies in cell lines and primary cells, especially in the CNS, are leading to many groups identifying candidate mechanisms and targets in the highly malleable system of cell lines, and then following these with studies in primary neurons. These follow up studies identify the fine mechanistic details in these highly specialized cell types. For example, cell lines overexpressing APP were used to demonstrate the inverse coupling of amyloidogenic and non-amyloidogenic processing. This simplistic model has recently been challenged as inhibition of endogenous BACE1 in primary neurons did not lead to increases in α-secretase

activity although inhibition of ADAM10 did lead to increased Aβ levels (Kuhn et al., 2010, Colombo et al., 2012). Furthermore, spatial regulation of APP processing was initially demonstrated in non-polarised Madin-Darby canine kidney (MDCK) cells where differential sorting of full length APP and its proteolytic products to the apical and basolateral membranes were observed (Haass et al., 1995a). This gave the first indication of two separate pathways of APP processing, a discovery crucial to AD research, however when studies were conducted in primary neurons, critical differences in signal peptide requirements for APP delivery to neuronal specific compartments were described (Back et al., 2007). These studies highlight the relative strengths of primary neurons in accurate replication of the fine modulation of processing that is not possible in cell lines. Such mechanisms of regulation have been vitally important discoveries in investigation of the physiological role of APP to better understand the pathophysiological effects its misregulation causes.

BACE1 inhibition using commercial inhibitors caused a maximal 50% reduction in luciferase expression, whilst DAPT caused a 75% reduction in luciferase expression. As the assay was insensitive to α-secretase inhibition, and even showed a small potentiation, it is worth considering the discrepancy between the DAPT and BACE1 inhibition levels. Although it is well accepted that BACE1 is the major, physiological β-secretase enzyme, cathepsin B, an endosomal/lysosomal cysteine protease has been proposed as an alternative minor β-secretase enzyme (Hook et al., 2005). Inhibition and genetic deficiency of cathepsin B have been shown to reduce Aβ levels in transgenic mice overexpressing WT APP (Hook et al., 2008, Hook et al., 2009), suggesting it plays a role in Aβ generation. Cleavage of APP by caspases has also been suggested to contribute to AD pathology. Specifically, caspase 3 was suggested to directly cleave APP following apoptotic stimuli contributing to increased Aβ secretion (Gervais et al., 1998). The discrepancy between BACE-sensitive and DAPT-sensitive APP processing events could therefore be due to non-secretase pathways of APP proteolysis such as the cathepsin B-mediated or caspase-3 dependent cleavage. Caspase activity is unlikely to be responsible as previous reports have shown the APP-Gal4 assay to be insensitive to caspase inhibition (Hoey et al., 2009) suggesting cathepsin is the more probable candidate.

### 3.3.2 Fe65 enhances the dynamic range of the APP-Gal4 luciferase assay in primary neurons

Due to the poor transfection efficiency of primary neurons, the level of luciferase expression was initially very low, making the differential between basal and stimulated conditions small. Co-expression of Fe65 enhanced luciferase expression, increasing the dynamic range of the assay. The role of Fe65 in modulation of APP processing is multifactorial and still not fully understood, however one of the first roles identified was as a stabiliser of the AICD. In the absence of Fe65 binding, the AICD is rapidly degraded (Kimberly et al., 2001, McLoughlin and Miller, 2008). Stabilising the AICD-Gal4 through co-expression of Fe65 and reducing the degradation rate of AICD, increases the number of APP processing events that lead to transactivation of the Firefly luciferase gene.

### 3.3.3 The APP-Gal4 assay is sensitive to pro-oxidant conditions in primary neurons

Following successful validation and characterisation of the APP-Gal4 assay, it was also shown to be sensitive to pathological changes associated with AD. Oxidative stress is intimately linked to AD, and is increased significantly earlier than Aβ burden in Down's syndrome patients (Nunomura et al., 2000), suggesting it to be one of the earliest events of the disease process, preceding the biochemical changes. AD brains exhibit multiple pathologies indicating increased oxidative stress including increased lipid peroxidation and increased AGEs (Wang et al., 2013). These changes were parallel to increased BACE1 expression and activity levels in vulnerable regions of post-mortem AD brain (Fukumoto et al., 2002, Yang et al., 2003).

In primary culture, media supplements are the major source of antioxidants. Chronic removal of antioxidants from the media at 1 DIV led to significant

enhancement of luciferase expression, suggestive of increased amyloidogenic processing or increased APP expression. This is consistent with findings showing oxidative agents and oxidative products such as $H_2O_2$ and 4-hydroxynonenal (HNE) causing increased BACE1 expression and activity in a concentration-dependent manner, consequently increasing amyloidogenic processing and Aβ production (Tamagno et al., 2002, Tamagno et al., 2008, Chami and Checler, 2012, Tan et al., 2013). Further to this, *in vivo* studies in mice have shown cortical levels of Aβ increase by 55% in animals fed a pro-oxidant diet compared with control, independent of changes in BACE1 expression implying redox sensitive changes in processing (Choudhry et al., 2012). This suggests that increased luciferase expression observed is more likely to be as a consequence of increased amyloidogenic processing rather than increased APP expression.

Much of the evidence for up-regulation of the amyloidogenic pathway in response to oxidative stress has come from studies involving high concentrations of $H_2O_2$, when apoptosis and cell death were also occurring. However, the oxidative stress model presented here showed no signs of apoptosis through morphological examination and there was no gross cell death, although loss of dendritic extensions was observed. A recent study showed that mild oxidative stress, induced by low concentrations of $H_2O_2$, caused increased amyloidogenic processing through relocalisation of BACE1 to the TGN and early endosomes. This increased the ratio of BACE1 in the acidic environment optimal for its activity and increased co-localisation of BACE1 with APP (Tan et al., 2013). It is therefore possible that chronic antioxidant withdrawal led to increased amyloidogenic processing through redistribution of BACE1 to cellular compartments favouring amyloidogenic processing although this is not something that has been explored. It cannot be discounted however, that apparent increases in amyloidogenic processing were due to increases in APP expression, thus increasing availability of the substrate causing a general increase in total APP processing.

Although no gross neuronal loss was observed following antioxidant withdrawal, a reduction in the dendritic network was noted. Activity-dependent APP processing is associated with potentiation of the non-amyloidogenic pathway (Hoey et al., 2009,

Verges et al., 2011); therefore a loss of dendritic connections and thus connectivity within the culture may reduce the activity input to oppose amyloidogenic APP processing, leading to increased levels of this pathway.

These alternative hypotheses are not mutually exclusive and could cumulatively lead to the increased luciferase expression seen in the assay. As levels of APP, BACE1, measurements of BACE1 activity and synaptic transmission were not conducted, it is not possible to confirm a particular mechanism in this system. Up-regulation of APP-Gal4 mediated luciferase expression showed the assay was sensitive to this known pathological insult that is strongly implicated in the development of AD. This lends additional indirect support to the conclusion that the assay is a measure of βγ-mediated APP processing.

### 3.3.4 The APP-Gal4 assay is sensitive to FAD-causing APP mutations

Development of an APP-Gal4 plasmid expressing the double point mutation (K670N/M671L) encoding the Swedish mutant of APP allowed a second model of pathological sensitivity to be assessed. The Swedish mutation of APP is a double substitution mutation immediately prior to the BACE1 cleavage site. It was identified in a Swedish familial AD (FAD) family and has been shown to produce 6-8 fold more Aβ than cells expressing normal APP (Citron et al., 1992). It is thought this increase may be due to changes in the cellular location of β-secretase cleavage, with APPswe BACE1 cleavage occurring within secretory vesicles, the same site as α-secretase activity, rather than later in the endosomal pathway as has been shown for normal BACE1 processing (Haass et al., 1995b). Therefore rather than α-secretase cleavage precluding BACE1 cleavage, there is direct competition between the two activities. This leads to increased levels of βγ-mediated APP processing products compared to WT. The higher levels of luciferase expression observed following transfection of neurons with APPswe-Gal4 compared with WT APP further supported the assay as a preferential readout of βγ-secretase-mediated APP

processing. Although transfection efficiencies were controlled for using an internal control plasmid, changes in APP expression levels between WT and mutant APP cannot be discounted as the cause of increased luciferase expression. As expression levels of both APP-Gal4 and APPswe-Gal4 proteins were too low for biochemical detection this concern could not be addressed.

### 3.3.5 Summary

This reporter assay is a sensitive and powerful approach for screening compounds with potential bioactivity at different points within the pathways regulating APP processing and is not simply a tool for identifying direct catalytic inhibitors of β and γ-secretase. Adopting the assay in a primary neuronal model represents a significant advantage over cell lines which lack the underlying neurophysiological context needed to draw meaningful conclusions about fine regulation.

# Chapter 4

## 4. *In vitro* screen identifies flavonoids as potent inhibitors of βγ-secretase mediated APP processing

# 4.1. Introduction

Epidemiological evidence collated over more than a decade, strongly suggests that there is a link between increased dietary flavonoid intake and reduced risk of developing AD. Foodstuffs including green tea, strawberries, blueberries, and fruit juices as well as adherence to a lifestyle such as the Mediterranean diet have been implicated in reducing risk of AD (Dai et al., 2006, Kuriyama et al., 2006, Feart et al., 2009, Devore et al., 2012). Transgenic mouse models of AD have been used to demonstrate that select flavonoids have beneficial effects on different aspects of AD pathology: Tg2576 mice orally administered EGCG, showed reduced amyloid pathology and improved cognitive function in a radial arm water maze test of working memory (Rezai-Zadeh et al., 2008). A flavonoid-rich GSPE orally delivered for 5 months also showed reduced incidence of high molecular weight Aβ oligomers and reduced amyloid plaque burden in adult Tg2576 mice (Wang et al., 2008). The flavonol myricetin, fed to Tg2576 mice for 10 months led to increased Aβ monomers, and concurrent decreased Aβ oligomers, suggesting a similarity to the GSPE-mediated oligomerisation reduction (Hamaguchi et al., 2009). IP injection of the flavone nobiletin for 4 months rescued memory impairment and reduced Aβ deposition in the hippocampus (Onozuka et al., 2008). Another flavone, diosmin, delivered by gavage for 6 months, reduced soluble Aβ, Aβ deposition and plaque burden (Rezai-Zadeh et al., 2009). The epidemiological evidence and *in vivo* studies suggest that flavonoids from across the families possess bioactivity at reducing amyloid beta pathology. However, the studies described thus far have been carried out on a chosen compound or small subset of compounds, investigating their effects in isolation, not in comparison. The flavonoid field has been held back by inconsistencies in findings and lack of a coherent, systematic approach across the families.

For this reason, this chapter describes the utilisation of the previously characterised and validated APP-Gal4 luciferase reporter assay in primary neurons to screen

flavonoids from across the families for modulatory activity on βγ-secretase mediated luciferase expression.

The flavonoids entered into the screen were selected based on a number of criteria:

1. previously published activity at APP such as the flavanol EGCG (Rezai-Zadeh, 2005);

2. high content in implicated foodstuffs such as cyanidin and pelargonidin which are major flavonoid constituents of berries (Devore et al., 2012);

3. activity at other relevant cellular targets such as fisetin, shown to activate ERK at 1 μM (Maher et al., 2006).

Further efforts were made to ensure the screen included a range of different flavonoid structures to enable basic structure activity relationships to be evaluated. Following initial identification of active compounds, more detailed concentration and kinetic analyses were carried out. Further analysis of efficacy was undertaken using the pathogenic APPswe-Gal4 system. In order to establish if flavonoid-mediated inhibition of βγ processing resulted in reductions in Aβ production an Aβ ELISA was performed on the media from flavonoid-treated neurons.

# 4.2. Results

## 4.2.1. Flavonoid screen identified potent inhibitors of βγ-mediated APP processing

Previous reports have suggested that flavonoids may be able to confer a positive effect on synaptic health and cognitive function by reducing Aβ levels through modulation of secretase-mediated APP processing (Obregon et al., 2006, Rezai-Zadeh et al., 2009, Wang et al., 2012). To date, however, no comparative study of flavonoid bioactivity on APP processing at a physiologically relevant concentration has been conducted. To address this, a library of flavonoids were selected and screened for modulatory effects on the APP-Gal4 gene reporter assay, described and characterised as a measure of βγ-secretase mediated APP processing in Chapter 3. The flavonoids were chosen from across the subfamilies with representative molecules from the flavones, flavonols, flavanones, flavanols and anthocyanins. Two concentrations of flavonoids were tested: 100 nM as a physiologically relevant concentration that has been measured in rodent brain following oral flavonoid delivery (van Praag et al., 2007, Wang et al., 2012), and at 10 μM, in line with previous reports of bioactivity of flavonoids in other studies (Rezai-Zadeh, 2005, Ehrnhoefer et al., 2008).

To assess flavonoid bioactivity on the APP-Gal4 reporter gene assay, primary neuronal cultures were transfected and 1h post transfection were treated with either 100nM or 10 μM flavonoid; levels of luciferase expression were measured 24h post flavonoid application. Relatively young cultures were used to maximize the transfection efficiency but were sufficiently mature to have formed synaptic connections in the cultures.

Four flavonoids inhibited APP-Gal4 dependent luciferase expression at 100 nM (Table 4.1): the flavanol epigallocatechin (57%), the flavonol fisetin (67%), the anthocyanin pelargonidin chloride (61%) and the flavone sinensetin (51%). Of these, epigallocatechin, fisetin and pelargonidin chloride inhibited at 100 nM but not at 10 μM. Epigallocatechin and fisetin (149% and 188% respectively) promoted luciferase expression at 10 μM. Sinensetin was inhibitory at both concentrations (51% and 57%). The structural variety in the identified molecules and the different patterns of inhibition at the two concentrations tested was suggestive of a number of different inhibitory mechanisms. It was interesting to note that no flavonoid was inhibitory at 10 μM but not at 100 nM. Only sinensetin, which was inhibitory at 100 nM also showed inhibitory action at 10 μM, suggesting flavonoids generally exert their beneficial effects on APP processing at low concentrations.

The catechins have been some of the most intensely researched flavonoids. This has been due to discovery that they can cross the blood brain barrier and that they show no toxicity to humans (Abd El Mohsen et al., 2002, Schroeter et al., 2010). Two structurally similar catechins to epigallocatechin that was identified in the original screen, (-) epicatechin and EGCG have previously been proposed to have activity against APP processing. It was therefore decided to focus on the catechin family to conduct a detailed concentration and kinetic analysis of their activity.

### 4.2.2. Epigallocatechin inhibits βγ-secretase dependent-luciferase expression

The flavanol epigallocatechin was identified in the original screen as an inhibitor of APP-Gal4 dependent luciferase expression at 100 nM but potentiated APP-Gal4 dependent luciferase expression at 10 μM. This suggested that inhibition of APP processing by the catechin family was strongly dependent on the concentration at which they were tested. To further investigate this, transfected primary cortical cultures were treated with epicatechin, epicatechin gallate, epigallocatechin or EGCG (10 nM-10 μM).

| | | % Activity Compared to Control | |
|---|---|---|---|
| Flavonoid group | Compound name | 100 nM | 10 µM |
| Flavonol | Fisetin[1] | 67.7±11.7 | 188±15.8 |
| | Kaempferol | 103±54.1 | 123±56.7 |
| | Kaempferol 3O Rutinoside | 83.0±44.7 | 85.8±19.7 |
| | Quercetin | 98.6±18.1 | 174±20.3 |
| Flavone | Apigenin | 90.9±27.9 | 154±17.0 |
| | Apigenin 7O Glucoside | 102±13.6 | 216±8.07 |
| | Coumarin | 137±15.1 | 139±27.4 |
| | Diosmetin | 90.4±14.3 | 322±14.1 |
| | Hyperoside | 94.1±14.3 | 108±17.9 |
| | Sinensetin[2'] | 51.1±26.1 | 57.6±18.2 |
| Flavanone | Hesperetin | 134±14.5 | 1378±52.2 |
| | Narirutin | 126±20.7 | 145±26.4 |
| Anthocyanin | Cyanidin Chloride | 111±39.8 | 95.5±30.5 |
| | Delphinidin Chloride | 114±32.9 | 200±5.92 |
| | Pelargonidin Chloride[3] | 61.4±6.23 | 98.9±16.4 |
| Flavanol | (+) Catechin | 97.6±14.7 | 105±10.4 |
| | (-) Epicatechin | 107±35.1 | 87.5±34.2 |
| | Epicatechin Gallate | 115±28.1 | 114±20.4 |
| | Epigallocatechin[4'] | 57.2±23.3 | 149±46.5 |
| | Epigallocatechin Gallate | 91.9±33.6 | 89.7±43.2 |

Table 4.1: **APP-Gal4 dependent-luciferase expression is modulated by select flavonoids in primary cortical neurons**. 5 DIV primary cortical neurons were co-transfected with 0.5 µg pRC-APP-Gal4, pFR-luciferase, pRL-TK-Renilla, after 0.5h, cells were treated with either 100 nM or 10 µM flavonoid compound for 24h. Cells were lysed and levels of luminescence quantified utilising the Dual-Glo luciferase kit according to manufacturer's instructions. [1-4] Flavonoids showing largest inhibition of luciferase expression. [2] and [4] caused significant reduction in luciferase expression at 100 nM (' $p<0.05$., One way ANOVA with Bonferroni post-test). Values expressed as mean ± S.D. (n=4).

The control level of luciferase expression achieved following transfection was very low. Co-transfection with the adaptor protein Fe65 increased luciferase expression levels significantly (Chapter 3). For this reason, parallel investigations of catechin efficacy were carried out following co-transfection with Fe65. This allowed assessment of the biological impact of Fe65 co-transfection on flavonoid modulation of APP-Gal4 dependent luciferase expression as it had been previously suggested that Fe65 decreased assay sensitivity (Hoey et al., 2009).

Firstly, comparison of the modulatory effects of the catechin family on APP-Gal4 dependent luciferase expression in either the presence or absence of Fe65 showed that Fe65 did not reduce assay sensitivity. 24h exposure to (-) epicatechin, epicatechin gallate or EGCG had no significant effect on luciferase expression at any concentration tested, either in the presence or absence of Fe65 (Figure 4.1(A, B and D)).

Epigallocatechin, in accordance with the initial screening data, showed bioactivity in the assay, with significant inhibition of luciferase expression at 100 nM (Figure 4.1(C)). Also consistent with the original screen was the lack of inhibition by epigallocatechin at higher concentrations. Following maximal inhibition at 100 nM, epigallocatechin showed no significant inhibition at higher concentrations and in the presence of Fe65 showed significant promotion of luciferase expression at 10 µM. This was consistent with the original screen. In contrast, in the absence of Fe65, no significant promotion of luciferase expression was observed (Figure 4.1(C)).

### 4.2.3. (-) Epicatechin inhibits reporter-dependent luciferase expression following 6h treatment

Flavonoids are quickly metabolised, with flavanol levels almost back to basal 8h following ingestion (Rein et al., 2000, Renouf et al., 2013). To investigate the time-dependence of catechin bioactivity on APP processing, a second, more acute treatment time of 6h was chosen. It was felt that this would better represent the *in*

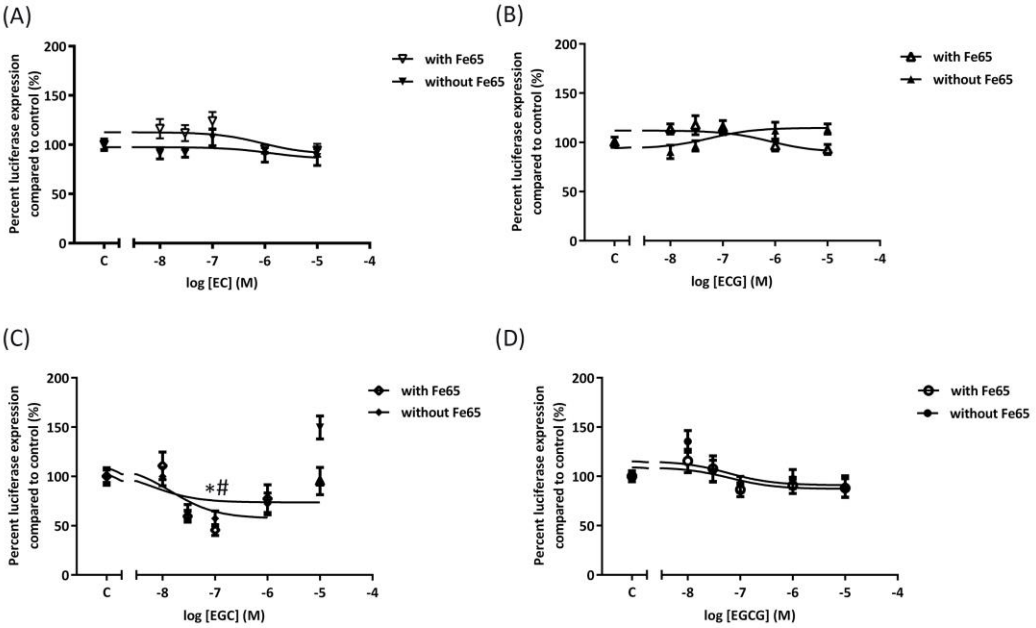



Figure 4.1: **Concentration analysis at 24h identified epigallocatechin as biphasic inhibitor of APP processing.**

5 DIV primary cortical neurons were co-transfected with 0.5 µg pRC-APP-Gal4, pFR-luciferase, pRL-TK-Renilla, after 0.5h, cells were treated with (A) EC - (-) epicatechin, (B) ECG - epicatechin gallate, (C) EGC - epigallocatechin and (D) EGCG -epigallocatechin gallate (0.01-10 µM) for 24h. In parallel, the same treatments were conducted following co-transfection with 0.5 µg pC1-Fe65, pRC-APP-Gal4, pFR-luciferase, pRL-TK-Renilla. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression and expressed as % control relative luminescence. Each point is the mean ± S.E.M of 17 independent transfections across 3 biological replicates (n=17; * without Fe65, # with Fe65, $p<0.05$, two way ANOVA with Bonferroni post-test).

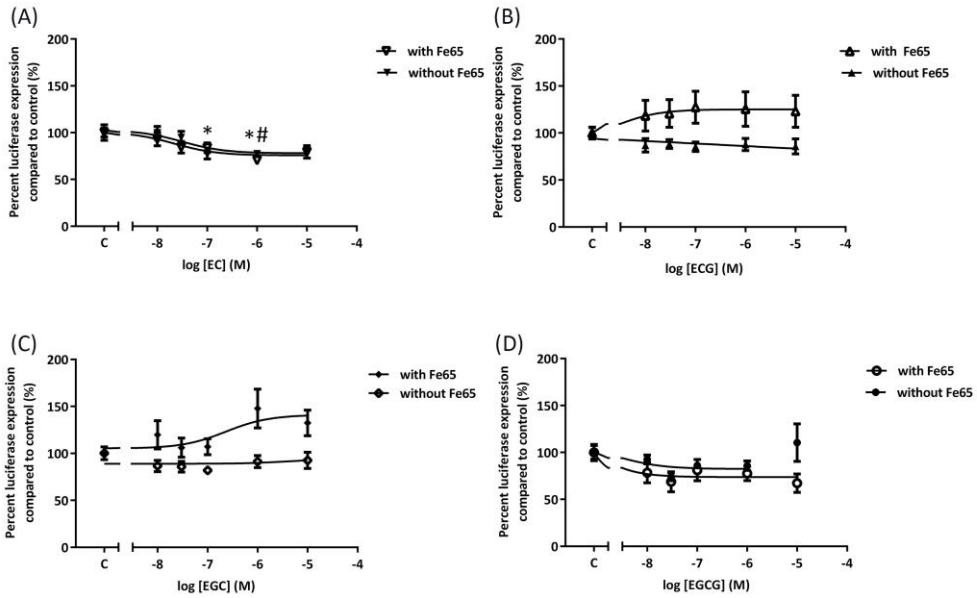



Figure 4.2: **Concentration analysis at 6h identified (-) epicatechin as inhibitor of APP processing.**

5 DIV primary cortical neurons were co-transfected with 0.5 µg pRC-APP-Gal4, pFR-luciferase and pRL-TKRenilla in the presence and absence of 0.5 µg pC1-Fe65. After 18h, transfected cells were treated with (A) EC - (-) epicatechin, (B) ECG - epicatechin gallate, (C) EGC - epigallocatechin and (D) EGCG -epigallocatechin gallate (0.01-10 µM) for 6h. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression and expressed as % control relative luminescence. Each point is the mean ± S.E.M. of 15 independent transfections across 3 biological replicates (n=15; * without Fe65, # with Fe65 $p<0.05$, two way ANOVA with Bonferroni post-test). )

*vivo* kinetics following flavonoid exposure. Cultures were treated with (-) epicatechin, epicatechin gallate, epigallocatechin or EGCG (10 nM-10 μM), either in the presence or absence of Fe65. Luciferase expression levels were measured 6h post catechin application.

Following 6h treatment, epicatechin gallate and EGCG had no significant effect on luciferase expression in either the presence or absence of Fe65, consistent with 24h treatment (Figure 4.2 (B and D)). In contrast, (-) epicatechin treatment, which was not inhibitory at 24h (Figure 4.1(A)), caused significant inhibition of luciferase expression at 6h (Figure 4.2 (A)). In addition, epigallocatechin, which was inhibitory after 24h treatment (Figure 4.1(C)), showed no significant inhibition of luciferase expression at 6h (Figure 4.2 (C)). These results indicated that the bioactivity of effective flavanols was time-dependent, suggesting potential differences in cell permeability or metabolism.

### 4.2.4. Transfection of Fe65 does not alter the biological efficacy of the catechins

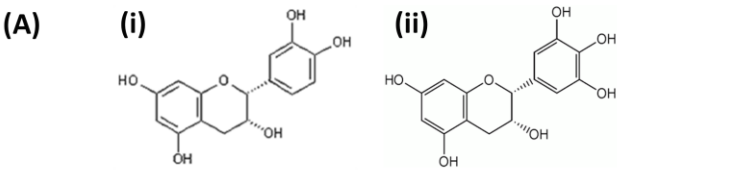
Transfection of Fe65 has previously been suggested to alter the sensitivity of primary neurons to signalling pathways associated with APP processing (Hoey et al., 2009). Due to low levels of transfection it was preferable for Fe65 to be utilised in the kinetic analyses of the catechins as Fe65 enhanced luciferase expression levels (Figure 3.6). To establish whether Fe65 had an impact on the efficacy or potency of the catechins, all experiments were performed in parallel either in the presence or absence of Fe65. In cultures transfected with Fe65, the pC1-Fe65 plasmid was added to the DNA transfection mix (see section 2.1.3.) and co-transfected with the required assay plasmids.

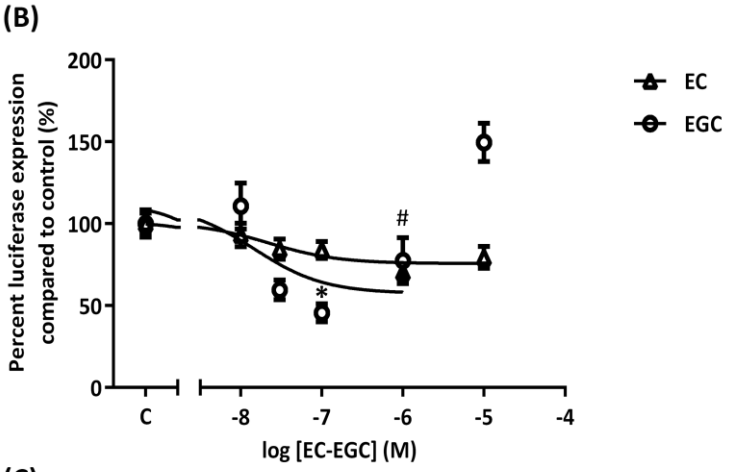
Epigallocatechin and (-) epicatechin were identified as inhibitors of APP-Gal4 dependent luciferase expression at 24h and 6h respectively both in the presence and absence of Fe65 (Figure 4.1 (A and C) and 4.2 (A and C)), supporting the conclusion that Fe65 does not alter the basic modulatory sensitivity of the assay.

Fe65 did however alter the concentrations at which inhibiton by the catechins reached statistical significance. (-) Epicatechin (6h) inhibition of luciferase expression was significant at both 100 nM and 1 μM in the absence of Fe65 but only at 1 μM in the presence of Fe65 (Figure 4.2(A)). Epigallocatechin (24h) treatment however was unaffected by Fe65, with significant inhibition of luciferase expression at 100 nM in both conditions. The biphasic effect of epigallocatechin, with promotion of luciferase expression at the highest concentration (10 μM) was less pronounced in the absence of Fe65, with a significant difference between the luciferase expression levels in the two conditions (Figure 4.1(C)). This is suggestive of a transcription-dependent mechanism for the epigallocatechin-mediated increase in APP processing.

### 4.2.5. (-) Epicatechin and epigallocatechin identified as potent inhibitors of APP processing.

Through the kinetic and concentration-dependent analyses, two effective flavanols were identified: (-) epicatechin and epigallocatechin. In order to make a direct comparison between them, their maximal concentration-responses were plotted against each other. Epigallocatechin was a more effective inhibitor, reducing luciferase expression to a maximum of 45% of control whilst (-) epicatechin showed a maximal inhibition of 70% of control. (Figure 4.3(B)). Despite the differences in the efficacy and kinetic of the responses, (-) epicatechin and epigallocatechin had similar potencies at their most effective time-point. (-) Epicatechin potently reduced luciferase expression ($EC_{50}$ of 22.1 nM) at 6h (Figure 4.3(C)), whilst epigallocatechin potently reduced luciferase expression ($EC_{50}$ of 14.3 nM) at 24h (Figure 4.3(C)). Epigallocatechin promoted amyloidogenic APP processing at 10 μM, therefore to calculate an accurate $EC_{50}$ value for its inhibitory action, this point was excluded from the curve fit. (-) Epicatechin showed no concentration-dependent effect at 24h (Figure 4.1(A)) whilst epigallocatechin showed no concentration-dependent inhibition at 24h (Figure 4.2(C)).




(C)

| | | Flavanol | |
|---|---|---|---|
| | Treatment length (h) | EC | EGC |
| $EC_{50}$ (nM) | 6 | 22.1 | N/A |
| | 24 | N/A | 14.3 |

Figure 4.3: **(-) Epicatechin and epigallocatechin are potent inhibitors of APP processing**.

**(A)** (i) (-) Epicatechin is a 3 ringed polyphenol with hydroxyl substitutions at position 7 of the A, ring 3 of C ring and 4', 5' of the B ring. (ii) Epigallocatechin has same core structure as (-) epicatechin with additional hydroxyl substitution at position 3' of the B ring. (B) Direct comparison of effective concentration responses (0.01-10 µM) of (-) epicatechin (6h) and epigallocatechin (24h) expressed as % control relative luminescence. EC- (-) epicatechin, EGC- epigallocatechin. (C) (-) epicatechin (6h) treatment potently inhibited βγ-secretase-dependent APP processing with an $EC_{50}$ of 20.5nM. Epigallocatechin treatment (24h) potently inhibited βγ-secretase-dependent APP processing with $EC_{50}$ of 18.6nM. Each point is the mean ± S.E.M. of 15-17 independent transfections across 3 biological replicates (n=15-17; * EGC $p<0.05$, # EC $p<0.05$, two way ANOVA with Bonferroni post-test).

### 4.2.1. (-) Epicatechin and epigallocatechin treatments show a trend towards inhibition of APPswe-Gal4 dependent luciferase expression

The APP-Gal4 screen identified two potent inhibitors of non-stimulated βγ-mediated APP processing. In order to test the efficacy of (-) epicatechin and epigallocatechin against a more pathological insult, primary cortical cultures transfected with the APP Swedish mutant APPswe-Gal4 (see Section 2.2.4) were treated with either (-) epicatechin for 6h or epigallocatechin for 24h (10 nM-10 μM). As a positive control, the γ-secretase inhibitor DAPT (10 μM for 24h) was included.

Consistent with results in Figure 3.9, transfection with APPswe-Gal4 increased luciferase expression compared to APP-Gal4 (control) levels and this expression was sensitive to DAPT inhibition (Figure 4.4 and 4.5).(-) Epicatechin and epigallocatechin treatments both showed trends towards reduced luciferase expression . The inhibitory trend was more apparent following epigallocatechin treatment however there was higher variability..

### 4.2.2. (-) Epicatechin treatment of primary cortical neurons suggests flavanol-mediated reduction in secretion of $A\beta_{x-40}$

Consistent inhibitory trends by (-) epicatechin and epigallocatechin of both the WT and Swedish APP-Gal4 dependent luciferase expression supported their bioactivity at reducing amyloidogenic APP processing. To further validate these findings and to establish if the reduced levels of processing observed in (-) epicatechin treated neurons resulted in lower levels of Aβ production an Aβ ELISA was used to directly measure levels of Aβ secreted into the media.

Primary cultured cortical neurons were treated with DAPT (10 μM) or vehicle for 24h to confirm that any Aβ measured in the media resulted from APP processing rather than unregulated protein secretion or cell death. (-) Epicatechin was applied to vehicle pre-treated neurons for the final 6h of incubation at two concentrations: 100 nM and 300 nM as a physiologically relevant concentration range that was effective in the APP-Gal4 luciferase assay. Following incubation, the media was removed and Aβ levels quantified from a standard curve. (-) Epicatechin treatment showed a concentration-dependent trend towards reduced Aβ levels; 300 nM (-) epicatechin treatment reduced mean Aβ levels to 43% of control (Figure 4.6(B)). Due to high variability of control levels of Aβ, this inhibition did not reach significance. Levels of Aβ following treatment with DAPT were negligible and therefore could not be extrapolated from the standard curve (Figure 4.6(A)), but confirmed that Aβ levels measured were as a result of γ-secretase mediated APP processing.

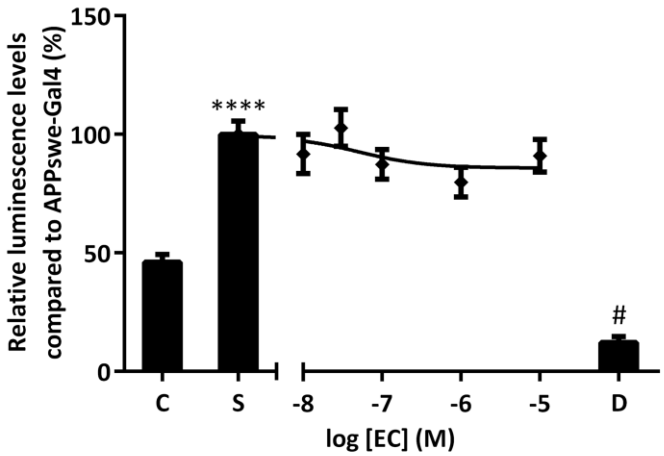



Figure 4.4: **(-) Epicatechin treatment causes a trend towards reduced pathogenic 'Swedish' mutant APP processing.**

5 DIV primary cortical neurons were transfected with either 0.5 µg pRC-APP-Gal4 or pRC-APPswe-Gal4; all wells were co-transfected with 0.5 µg each of pFR-luciferase, pRL-TK-Renilla and pC1-Fe65. 0.5h later pRC-APPswe-Gal4 transfected neurons were treated with DAPT (10 µM). 18h post-transfection pRC-APPswe-Gal4 transfected neurons were treated with (-) epicatechin (0.01-10 µM) for 6h. Co-transfection of pRC-APPswe-Gal4 significantly increased relative luciferase expression compared to pRC-APP-Gal4. Treatment with DAPT significantly inhibited APPswe-Gal4 dependent luciferase expression. (-) Epicatechin potently inhibited βγ-secretase dependent APP processing with an $EC_{50}$ of 51 nM. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression and expressed as % APPswe-Gal4 control relative luminescence. Each column or point is mean ± S.E.M. of 24 independent transfections across 4 separate cultures (n=24 **** compared to C (APP-Gal4 control) $p<0.0001$ # compared to S (APPswe-Gal4 control) $p<0.0001$ One way ANOVA with Bonferroni post-test). D- DAPT (10 µM), EC – (-) epicatechin.

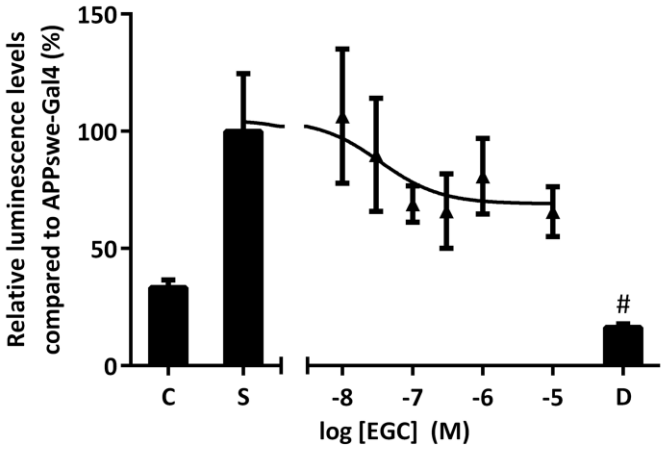



Figure 4.5: **Epigallocatechin treatment shows an inhibitory trend on pathogenic 'Swedish' mutant APP processing**

5 DIV primary cortical neurons were transfected with either 0.5 µg pRC-APP-Gal4 or pRC-APPswe-Gal4; all wells were co-transfected with 0.5 µg each of pFR-luciferase, pRL-TK-Renilla and pC1-Fe65. 0.5h later pRC-APPswe-Gal4 transfected neurons were treated with DAPT (10 µM) or epigallocatechin (0.01-10 µM) for 24h. Treatment with DAPT significantly inhibited APPswe-Gal4 dependent luciferase expression. Epigallocatechin potently inhibited βγ-secretase dependent APP processing with an $EC_{50}$ of 34 nM. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and *Renilla* luciferase expression. Firefly luciferase reporter expression was normalised using the *Renilla* luciferase expression and expressed as % APPswe-Gal4 control relative luminescence. Each column or point is mean ± S.E.M. of 25 independent transfections across 5 separate cultures (n=25, # compared to S (APPswe-Gal4 control) $p<0.0001$ One way ANOVA with Bonferroni post-test). D- DAPT (10 µM), EGC – epigallocatechin.

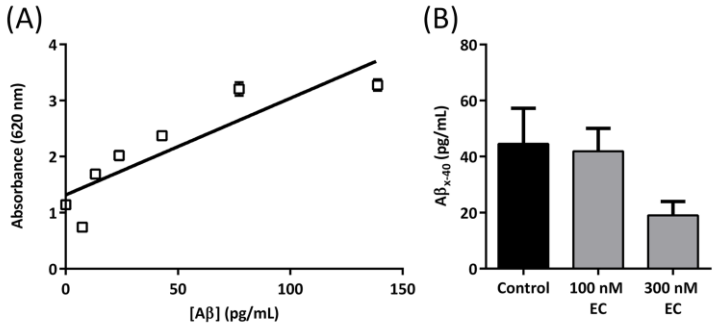



Figure 4.6: **Trend towards concentration dependent (-) epicatechin inhibition of $A\beta_{x-40}$ release into media in primary cortical neurons.**

(A) Standard curve was generated through serial dilution of $A\beta_{1-40}$ standard (n=3). (B) 5DIV primary cortical neurons were treated with vehicle (24h), 100 nM (-) epicatechin, 300 nM (-) epicatechin (6h) or 10 µM DAPT (24h). Aβ levels were assessed by ELISA using Colorimetric BetaMark x-40 ELISA (Covance). DAPT levels were negligible and therefore could not be estimated from standard curve. Each column is mean ± S.E.M of 3 independent cultures (n=3).

# 4.3. Discussion

The APP-Gal4 gene reporter assay was used to screen flavonoids leading to identification of four effective molecules that reduced levels of βγ-secretase dependent APP processing: epigallocatechin, fisetin, pelargonidin chloride and sinensetin. Further concentration and kinetic analyses of the catechin family of flavanols, chosen due to their favourable bioavailability and blood brain barrier permeability, identified (-) epicatechin, as well as confirming epigallocatechin, to be potent inhibitors of APP-Gal4 dependent luciferase expression. When challenged with more pathological levels of amyloidogenic processing, both (-) epicatechin and epigallocatechin remained potent inhibitors, although levels of inhibition were lower compared to the inhibition observed with WT APP. Finally, to validate the findings of the APP-Gal4 gene reporter assay, Aβ levels from the media of (-) epicatechin treated cultures were measured and showed reduction in Aβ levels.

## 4.3.1. APP-Gal4 gene reporter assay identified inhibitors of βγ-secretase mediated APP processing

The APP-Gal4 gene reporter assay identified four flavonoids with inhibitory actions at 100 nM. Only the flavone sinensetin was inhibitory at 10 µM, whilst a number of flavonoids promoted βγ-secretase processing at 10 µM. Epigallocatechin and fisetin showed bi-phasic effects inhibiting APP processing at 100 nM and stimulating APP processing at 10 µM. This biphasic profile has been reported previously for flavonoid modulation of ERK and Akt signalling pathways in neurons (Schroeter et al., 2007, Vauzour et al., 2007) and suggests that concentration is a critical determinant of flavonoid selectivity.

The four flavonoids identified from the screen each originate from a different flavonoid subfamily. As flavonoids are segregated into families dependent on their structure, this suggests there is not a simple structural link between flavonoids and

predicted bioactivity at APP. Traditionally, flavonoids were suggested to exert their beneficial effects through their activities as antioxidants, however antioxidant capacity of flavonoids is heavily dependent on their structure, therefore it is unlikely that this property of flavonoids was the mechanism responsible in reducing amyloidogenic processing. The concentrations at which the flavonoids exerted their effects also supports this conclusion, as it would be highly unlikely that nanomolar concentrations of flavonoids would exert a strong enough antioxidant effect to be beneficial over endogenous and media supplemented antioxidants such as vitamin E and ascorbate.

The identification of fisetin as an inhibitor of βγ-secretase mediated APP processing was consistent with previous work showing fisetin to be effective at reducing aspects of Aβ pathology. Fisetin has been shown to inhibit $A\beta_{1-42}$ fibrillogenesis *in vitro* and long term oral administration of fisetin to APP/PS1 transgenic mice improved spatial memory and reduced soluble Aβ. This is suggestive of an ability to affect APP processing (Kim et al., 2005a, Currais et al., 2013) and provides strong support for the APP-Gal4 screen as an approach for identifying bioactive molecules. Fisetin has also been shown to activate signalling pathways associated with long-term memory. Fisetin activated CREB in an ERK dependent manner, leading to increased LTP, the cellular mechanism highly implicated as crucial for memory formation. Similar to the fisetin-mediated inhibition of APP processing, LTP potentiation by fisetin was also a biphasic effect, maximal at 1 µM and lost at 10 µM (Maher et al., 2006). Although not directly comparable, the concentration dependence of fisetin-mediated inhibition in this study and correlation with previous studies is suggestive of it acting through cell signalling pathways.

Pelargonidin as a major flavonoid constituent of blueberries and strawberries has been implicated in reversing age-related cognitive decline (Joseph et al., 1999). Despite these promising results from epidemiological studies, no study has been published showing pelargonidin to be effective at reducing APP pathology. Thus, this is the first report of pelargonidin activity at APP. Although there was no previous evidence of pelargonidin being effective in AD, pelargonidin pre-treatment has been shown to be neuroprotective against neuronal injury induced by injection

of 6-hydroxydopamine and endogenous neurotoxins associated with PD (Vauzour et al., 2008, Roghani et al., 2010) providing additional evidence of activity in neuronal cell types. For flavonoids to be able to exert a positive effect on APP processing *in vivo,* the major hurdles of bioavailability and blood brain barrier permeability must be considered and overcome. Anthocyanidins are water soluble molecules and the pelargonidin aglycone reaches detectable levels in the brain following oral gavage, although it was shown to undergo rapid metabolism (El Mohsen et al., 2006). Pelargonidin may therefore be a structure of interest for drug development of APP processing modulation, despite the lack of mechanistic and efficacy data.

Sinensetin was the only flavonoid that inhibited APP-Gal4 mediated luciferase expression at both concentrations tested. Like fisetin, sinensetin has been shown to promote LTP-associated signalling pathways, however unlike fisetin which only potentiated at concentrations up to 1 µM, sinensetin has been shown to activate CRE-mediated transcription in rat hippocampal neurons, at 100 µM(Kawahata et al., 2013). It would therefore have been interesting to investigate the concentration-dependence of the sinensetin-mediated inhibition of APP-Gal4 mediated luciferase expression as this apparent lack of concentration-dependence is currently suggestive of an independent mechanism of APP-Gal4 dependent luciferase expression for sinensetin compared to the other flavonoids.

Epigallocatechin was perhaps the most significant positive hit from the assay, as it is a member of an intensively studied family of flavanol molecules called the catechins. The catechins have known bioavailability in a variety of mammalian models (van Praag et al., 2007, Ho et al., 2013) and have been previously postulated to have therapeutic potential (Williams and Spencer, 2012). For these reasons, further investigation of the catechin family was undertaken. Due to poor bioavailability (fisetin and sinensetin) and paucity of previous studies (pelargonidin and sinensetin) further investigation into these molecules was not carried out.

## 4.3.2. Flavanol inhibition of βγ-secretase-mediated APP processing is concentration and time-dependent

Through the initial screen, flavonoid concentration was shown to be critical to bioactivity of the flavonoids. It is also documented that flavonoids are quickly metabolised, potentially altering their structure into inactive forms. For these reasons, the catechin family were screened further over a wider range of concentrations and at two time points – 6h and 24h. Further concentration and kinetic analysis of the catechin family revealed that (-) epicatechin, in addition to epigallocatechin, possessed potent inhibitory actions but only when applied for shorter time periods.

Both (-) epicatechin and epigallocatechin showed maximal inhibition in the high nanomolar to low micromolar concentration range with neither causing inhibition at 10 μM. Epigallocatechin promoted amyloidogenic processing at the highest concentration tested, 10 μM and therefore this data point had to be excluded to enable accurate calculation of the $EC_{50}$ value of its inhibitory action. Calculation of $EC_{50}$ values for the activation of amyloidogenic processing by epigallocatechin was not possible due to a lack of data points at higher concentrations. The reason for this concentration dependent effect was not fully elucidated however may be linked to flavonoid activity at additional cellular targets at higher concentrations. Activation of PKC-dependent signalling pathways has been well characterised as a cellular mechanism for promotion of non-amyloidogenic processing (Mills and Reiner, 1999), causing a shift away from amyloidogenic APP processing (Hung et al., 1993). A concentration dependent inhibition of PKC by micromolar concentrations of flavonoids has previously been reported (Ferriola et al., 1989). Although the study did not specifically identify epigallocatechin or (-) epicatechin, inhibition of PKC-dependent-α-secretase activity and resultant increases in the level of amyloidogenic processing could counteract the inhibitory activities of (-) epicatechin and epigallocatechin at lower concentrations. This would lead to the apparent loss of inhibitory activity at 10 μM. Activity at PKC could also be

responsible for the promotion of amyloidogenic APP processing seen with epigallocatechin at 10 μM and would suggest that epigallocatechin was a more effective inhibitor of PKC-dependent signalling than (-) epicatechin.

The different kinetics of the catechins is difficult to address as previous studies have not investigated time-dependence of flavonoid activities. Epigallocatechin showed effective, potent inhibition of βγ-dependent APP processing at 24h but showed no activity at 6h. As cells were transfected for the same length of time for both the 6h and 24h treatments, it is unlikely to be due to insufficient luciferase expression levels in the 6h treatment. The gallate group of epigallocatechin not only increases its size but also increases its polarity, two factors that will reduce its cell permeability and increase the time taken for epigallocatechin to reach its cellular target, which could be a simple explanation for the time-dependence of the epigallocatechin effect. A second explanation could point to a transcriptional mechanism of action, whereby a 6h treatment does not provide enough time for epigallocatechin to exert its beneficial effect. Thirdly, flavonoids are known to be heavily metabolised, as no measurements of catechin metabolites in treated cultures have been made, it may be that epigallocatechin is not the bioactive form and cellular metabolism must first occur to generate the active molecule which can then exert its beneficial effect, once again, delaying the time at which the epigallocatechin treatment exerts its effect.

(-) Epicatechin exhibited the inverse time-dependence of epigallocatechin, showing inhibitory activity at 6h, but no activity at 24h. *In vivo* studies in rats and humans, measuring catechin absorption levels consistently identify the (-) epicatechin aglycone as a major constituent of the total catechin absorbed. This suggests that it is highly cell penetrant and therefore can reach intracellular targets rapidly to exert its effect (Rein et al., 2000, Spencer et al., 2001, Schroeter et al., 2006). Further bioavailability studies of (-) epicatechin show it reaches maximal concentrations 2h post ingestion and levels are reduced back to basal by 6-8h, suggesting it is rapidly broken down (Rein et al., 2000). The acute effect seen with (-) epicatechin is therefore likely due to breakdown of the active compound. The acute nature of the (-) epicatechin effect is also suggestive of a post-translational mechanism of action

that is not maintained once (-) epicatechin is degraded. It would have been of interest to study the effect of 24h (-) epicatechin treatment with repeated doses to investigate whether the lack of 24h activity of (-) epicatechin was due to active compound degradation.

Although (-) epicatechin and epigallocatechin showed similar potencies, (-) epicatechin was less effective than epigallocatechin. To ensure sufficient luciferase expression, cultures for the 6h catechin treatments were transfected 24h prior to luminescence detection. Therefore APP-Gal4 cleavage had been taking place for 19h prior to treatment with the specific catechin molecule. The measurement of luciferase expression therefore contained a proportion of expression that was not modulated by (-) epicatechin which would lead to an underestimate of the efficacy of (-) epicatechin inhibition.

### 4.3.3. Fe65 does not alter APP-Gal4 gene reporter assay sensitivity to modulators of APP processing

One of the major limitations of the APP-Gal4 gene reporter assay in primary neurons was the transfection efficiency and the related low level of luciferase expression. In order to extend the dynamic range of the assay, the adaptor protein Fe65 was co-transfected and led to significant induction of luciferase expression, most likely due to stabilisation of the γ-secretase cleavage product AICD-Gal4. However, it had been suggested that introduction of Fe65 would alter the biological effect of novel modulators in the screen and therefore all concentration and kinetic analyses of the catechin family were carried out in parallel in the presence and absence of Fe65 (Hoey et al., 2009).

In both the presence and absence of Fe65, epigallocatechin (24h) and (-) epicatechin (6h) were identified as potent inhibitors of APP-Gal4 dependent luciferase expression, supportive of Fe65 having no biological effect on modulation of APP by the catechin family.

### 4.3.4. (-) Epicatechin and epigallocatechin show inhibitory trends on pathogenic βγ-secretase-mediated APP processing

Previous studies have shown that certain flavanols reduce Aβ pathology in transgenic models of AD (Rezai-Zadeh, 2005, Wang et al., 2012). To determine whether (-) epicatechin and epigallocatechin were effective against higher, more pathological levels of βγ-mediated APP processing, primary neurons transfected with APPswe-Gal4 were treated with (-) epicatechin (6h) and epigallocatechin (24h). In agreement with the *in vivo* studies, (-) epicatechin and epigallocatechin showed inhibitory trends on APPswe-Gal4 luciferase expression.

The Swedish mutation of APP causes increased amyloidogenic processing through a relocalisation of amyloidogenic APP processing activity from the TGN and endosomes to secretory vesicles, the same compartment as the non-amyloidogenic APP processing. This increases competition at this site and increases the proportion of amyloidogenic APP processing (Haass et al., 1995b). The reduced efficacy of the catechins in the Swedish mutant assay may suggest that cellular localisation of APP is an important factor in catechin-mediated inhibition of βγ-mediated processing, however further investigation would be required to provide conclusive evidence for this. Analysis of catechin inhibition of other APP mutants associated with AD would further aid elucidation of the mechanism of action due to the range of mechanisms through which they increase Aβ pathology. This would allow assessment of flavanol efficacy at mutants reported to increase fibrillogenesis of Aβ, and mutants that alter cleavage site preference of the β- and γ-secretase activities.

## 4.3.5. (-) Epicatechin treatment shows inhibitory trend in production of $A\beta_{x-40}$

Due to the limitations of the luciferase assay, it was important to validate its findings using a different technique. Therefore the effect of (-) epicatechin (6h) treatment on Aβ production by ELISA was performed. As the concentration of $A\beta_{42}$ released from primary neuronal cultures is below the sensitivity level of current commercial ELISA kits, an Aβ ELISA measuring concentrations of released $A\beta_{x-40}$ was used.

(-) Epicatechin caused a maximal reduction of $A\beta_{x-40}$ of 43% of control, supporting the results from the APP-Gal4 luciferase gene reporter assay. This was also in agreement with an independent study that showed monomeric catechins, of which (-) epicatechin is a significant component, reduced $A\beta_{1-40}$ levels by approximately 50%, following chronic oral administration to transgenic Tg2576 mice (Wang et al., 2012). In collaborative work, we also showed that (-) epicatechin reduced Aβ levels in primary TASTPM cortical cultures by 30% and reduced Aβ plaque load by approximately a third following oral delivery for 21 days (Cox et al., 2014 under review).

## 4.3.6. Summary

This screen has identified potent inhibitors of amyloidogenic APP processing in primary neurons. Consideration of bioavailability and proven drug-like properties focussed study on the catechin family. (-) Epicatechin and epigallocatechin were potent inhibitors of amyloidogenic processing under physiological and pathophysiological conditions. Previous reports and collaborative data showed (-) epicatechin to be effective at reducing Aβ levels *in vitro* and *in vivo* in rodent models of AD, therefore the next chapter shall focus on (-) epicatechin to investigate the mechanisms through which it is exerting its inhibitory effect.

# Chapter 5

## 5. Mechanistic investigation of (-) epicatechin-mediated inhibition of amyloidogenic APP processing

# 5.1. Introduction

The APP-Gal4 reporter assay and further kinetic analyses identified epicatechin as a potent inhibitor of βγ-secretase mediated APP processing (Chapter 4). (-) Epicatechin reduced luciferase expression in the APP-Gal4 and APPswe-Gal4 reporter assays and also showed a trend toward reduced Aβ levels.

The processing of APP can follow the amyloidogenic or the non-amyloidogenic pathway. Differentiating these pathways is the initial shedding step, mediated by β-secretase in the amyloidogenic pathway and by α-secretase in the non-amyloidogenic pathway. Cleavage of APP by β-secretase releases the sAPPβ ectodomain and leaves the C-terminal stub, a 99 amino acid peptide (C99) retained in the membrane (Haass and Selkoe, 1993, Vassar et al., 1999). Cleavage of APP by α-secretase mediates release of sAPPα and retention of an 83 amino acid (C83) C-terminal stub in the membrane (Sisodia, 1992a, Lammich et al., 1999). The second cleavage step is common to both pathways, and is mediated by the γ-secretase, which releases the 49 amino acid AICD. This cleavage occurs in different cellular compartments depending on the processing pathway: at the plasma membrane for non-amyloidogenic and predominantly in endosomes for the amyloidogenic pathway (St George-Hyslop and Schmitt-Ulms, 2010). Due to the different N-termini of the C-terminal stubs, γ secretase mediates release of Aβ in the amyloidogenic pathway, and the 16 amino acid shorter P3 peptide in the non-amyloidogenic pathway (Lichtenthaler et al., 2011). Due to the differences in size and amino acid sequence at the C- and N-termini of the APP metabolites, they can be differentiated from each other using specific antibodies or size separation. This allows changes in APP processing to be deduced through measurement of specific APP metabolite levels.

Despite intense research into the potential cellular mechanisms through which flavonoids mediate risk reduction of AD and modulation of APP pathology, there is still great uncertainty as to how they are exerting their effects. The best-

characterised mode of action has been for the flavanol EGCG. Evidence from cell lines as well as primary transgenic neurons suggested that EGCG at micromolar concentrations enhanced maturation of ADAM10, leading to increased sAPPα and αCTF production and decreased Aβ levels (Rezai-Zadeh, 2005, Obregon et al., 2006). Although not supporting the ADAM10 involvement, independent studies have supported EGCG mediated increases in sAPPα in both cell lines (Levites et al., 2003) and primary neurons (Wang et al., 2006). Despite this well characterised mechanism for EGCG, it is still not clear-cut and other modes of action have been proposed. EGCG has also been shown to suppress Aβ-induced BACE1 up-regulation (Shimmyo et al., 2008a), to stabilise oligomers so that they do not stimulate fibrillogenesis (Ehrnhoefer et al., 2008) and to inhibit APP interactions with adaptor proteins (Lin et al., 2009).

Although not as well defined, an alternative mechanism for flavonoid modulation of APP processing is as direct catalytic inhibitors of BACE1 (Shimmyo et al., 2008b). Two flavones, quercetin and myricetin (20 μM), decreased BACE1 activity and decreased $A\beta_{1-40}$, and $A\beta_{1-42}$ levels in primary neurons. Inhibition of BACE1 provides the most direct mechanism for flavonoid-mediated inhibition of amyloidogenic processing; however the concentrations at which these flavonoids showed activity are not achievable in the brain, suggesting this activity may not be physiologically relevant. *In silico* docking studies suggest that flavonoids can directly interact with the active site of BACE1 through hydrogen bonds (Shimmyo et al., 2008b). *In silico* work allows analysis of potential interactions between proteins and thus can give useful mechanistic information once direct interaction has been experimentally established, however as this has not been conclusively proven, this catalytic inhibition mechanism remains speculative.

Flavonoid-mediated γ secretase modulation has also been proposed: another flavone, luteolin (20-40 μM), was shown to promote presenilin phosphorylation by GSK3α. This decreased γ secretase activity on APP through disruption of APP-PS1 association, thus decreasing Aβ production (Rezai-Zadeh et al., 2009). Pharmaceutical attempts to target γ secretase through traditional drug design have failed due to off-target actions at other γ secretase substrates notably notch.

Further considerations include potential toxicity due to accumulation of β-CTFs; however *in vitro* evidence suggests the build-up of β-CTFs is much lower following luteolin treatment than following inhibition by a direct γ secretase inhibitor. Indirect γ secretase inhibition may therefore be a less toxic, more regulative mode of inhibition (Rezai-Zadeh et al., 2009).

The complexity of APP processing regulation and the variety of flavonoids reported to have activity has made elucidation of a common mechanistic pathway extremely difficult. It was therefore important to try and elucidate the mechanism through which (-) epicatechin was exerting its effect in our system.

In order to determine the mechanism underlying the (-) epicatechin-mediated changes, biochemical analysis of APP metabolites in primary cortical neurons and cell lines were measured with particular focus on sAPPα modulation. Further to this, a recombinant BACE1 activity assay and a gene reporter notch assay were utilised to assess the involvement of the β- and γ-secretase enzymes.

# 5.2. Results

## 5.2.1. Notch activity is unaffected by epicatechin treatment

To investigate whether (-) epicatechin was acting through γ-secretase a notch-Gal4 luciferase reporter assay was used. Transfected primary cortical neurons were treated with epicatechin (0.1-10 μM) or DAPT (10 μM) for 6h and luciferase expression measured. DAPT caused a substantial reduction in luciferase expression demonstrating that the assay was sensitive to γ-secretase cleavage of notch as expected. (-) Epicatechin did not affect luciferase expression at either concentration (Figure 5.1); strongly suggesting its bioactivity was not at the level of γ-secretase.

## 5.2.2. (-) Epicatechin does not affect $APP_{695}$ levels

Chapter 4 identified (-) epicatechin and epigallocatechin as potent inhibitors of APP processing. Although these results were consistent with inhibition of βγ-secretase mediated APP processing there were a number of potential mechanisms through which this might be occurring. Changes in levels of $APP_{695}$ could lead to altered APP processing. If levels were reduced, less APP would be available for processing which would lead to apparent inhibition of APP processing in the APP-Gal4 assay. This would however be due to lack of substrate rather than modulation of processing itself. To investigate whether the inhibitory actions of (-) epicatechin were the result of changes in APP expression, primary neurons were treated with 100 nM (-) epicatechin over 24h and $APP_{695}$ levels were measured by western blot analysis. No significant change was observed (Fig 5.2(A and E)), indicating (-) epicatechin was not acting to down-regulate APP levels in primary neurons either through inhibition of translation or increased protein turnover.

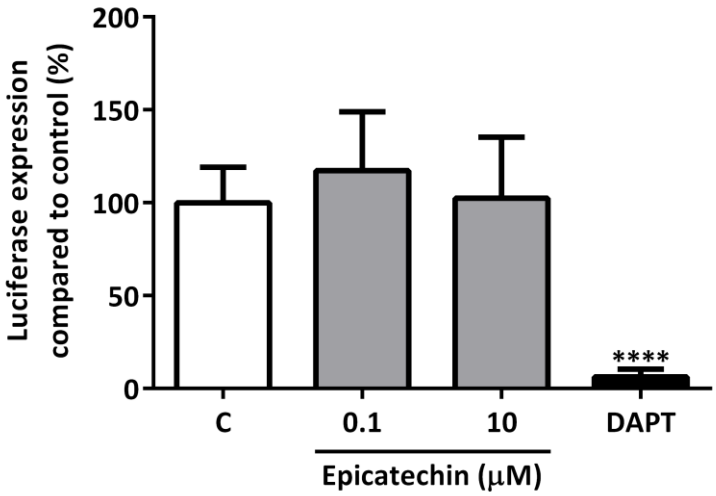



Figure 5.1: **(-) Epicatechin does not affect γ-secretase activity**

5 DIV primary cortical neurons were co-transfected with 0.5μg each Notch-Gal4, pFR-luciferase and pRL-TK-Renilla, after 0.5h and 18h, cells were treated with DAPT or (-) epicatechin (0.1 and 10 μM) respectively. Dual-Glo luciferase activity assays were performed 24h post transfection for quantification of firefly and Renilla luciferase expression. Firefly luciferase reporter expression was normalised using the Renilla luciferase expression and transformed to % control relative luminescence. Each column is the mean ± S.E.M. of 18 independent transfections from 3 separate cultures (n=18, **** $p<0.0001$ One way ANOVA with Bonferroni post-test).

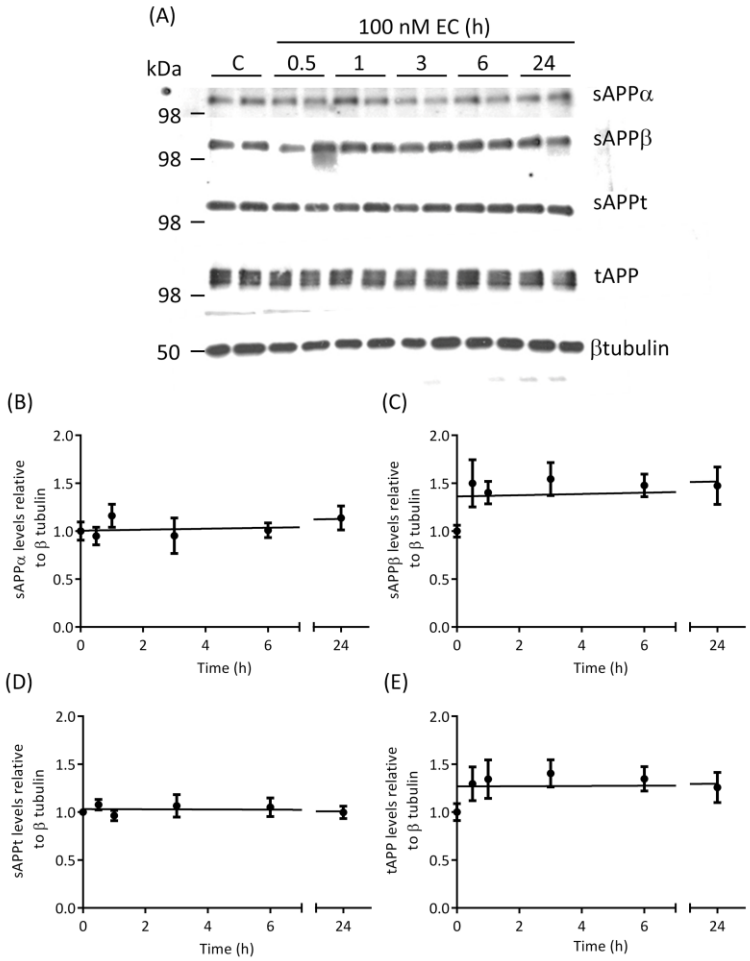



Figure 5.2: **(-) Epicatechin effects are independent of APP695 expression levels and ectodomain shedding in primary cortical neurons**

(A) 6 DIV primary C57/BL6 cortical neurons were treated with vehicle (C) or 100 nM (-) epicatechin (EC) for 30 min, 1h, 3h, 6h or 24h followed by immunoblotting with 22C11 (APP and sAPPt), 5G11 (sAPPα), 192WT (sAPPβ) or β tubulin. sAPPα, sAPPβ and sAPPt levels were measured from conditioned media removed from the neurons. $APP_{695}$ and β tubulin levels were measured from whole cell lysate. Levels of (B) sAPPα, (C) sAPPβ, (D) sAPPt and (E) tAPP relative to β tubulin were analysed by ECL protein band densitometry using calibrated Image J software. Each point represents the mean ± S.E.M. Representative blots from four independent experiments (n=4).

### 5.2.3. (-) Epicatechin does not affect sAPP levels in primary neurons

Previous studies investigating the effects of flavanols on APP processing have suggested up-regulation of the constitutive α-secretase enzymatic activity, ADAM10, as the likely mechanism of action (Obregon et al., 2006, Fernandez et al., 2010). Increased ADAM10 activity leads to increased levels of the neuroprotective, sAPPα. Furthermore, up-regulation of ADAM10-mediated APP processing has been shown to down-regulate production of amyloidogenic proteolytic products (Rezai-Zadeh, 2005, Colombo et al., 2012). In order to investigate this possibility, sAPPα levels in the culture medium following 100 nM (-) epicatechin treatments over 24h were measured using a sAPPα specific antibody by western blot analysis. (-) Epicatechin showed no time-dependent changes in the levels of sAPPα (Figure 5.2(A and B)) suggesting that α-secretase was not the primary target. This was supported by measurement of total secreted APP levels, using an APP N-terminal antibody, which also showed no change (Figure 5.2(A and D)).

Despite the literature favouring modulation of α-secretase as the mechanism of action of flavanols, the most direct mechanism through which (-) epicatechin exerts its inhibitory effects would be inhibition of BACE1-mediated APP processing. To investigate this, parallel measurements of sAPPβ expression levels following (-) epicatechin treatment were measured. (-) Epicatechin treatment caused no time-dependent changes in the levels of sAPPβ (Figure 5.2(A and C)) suggesting that BACE1 cleavage of APP was also unaffected.

### 5.2.4. (-) Epicatechin does not significantly affect APP or sAPPα production in SH-SY5Y or HEK293T cells

Unlike most cell types and immortalised cell lines, which display non-amyloidogenic processing as their dominant APP processing pathway, primary neurons exhibit a

dominant amyloidogenic pathway, accounting for over 50% of the APP processed in the cell (Colombo et al., 2012). To investigate whether cell type specificity would affect the ability of (-) epicatechin to modulate sAPPα levels, two cell lines, HEK293T and SH-SY5Y cells were treated with (-) epicatechin (0.1-10 μM) for 3h and 6h. sAPPα and $APP_{695}$ levels were then measured by western blot analysis (Figure 5.3 and 5.4). Both HEK293T and SHSY5Y showed a slight trend towards a time-dependent increase in sAPPα levels following (-) epicatechin treatment, however this did not reach significance (Figure 5.3(A-B) and 5.4(A-B)). $APP_{695}$ levels showed no significant change in either cell type (Figure 5.3(A and C) and 5.4(A and C)).

### 5.2.5. Sheddase enzyme expression is unaltered following (-) epicatechin treatment

APP metabolites provide an indication as to the levels and relative contributions of the two APP processing pathways. An alternative to measurement of the APP proteolytic products is to study expression levels of the secretase enzymes. Although this does not give a direct indication of the activity level of these enzymes, which is tightly controlled post-translationally, it allows (-) epicatechin mediated expression changes to be evaluated.

Measurements of ADAM10 and BACE1 expression levels following (-) epicatechin treatment were conducted. Primary neurons were treated with 100 nM (-) epicatechin for 0.5h and 6h and levels of ADAM10 and BACE1 in cell lysate measured by western blot analysis. Neither ADAM10 nor BACE1 expression levels were altered at either time point (Figure 5.5). As ADAM10 and BACE1 are the secretase enzymes primarily responsible for APP ectodomain shedding this result suggests that (-) epicatechin does not act as a regulator of sheddase expression.

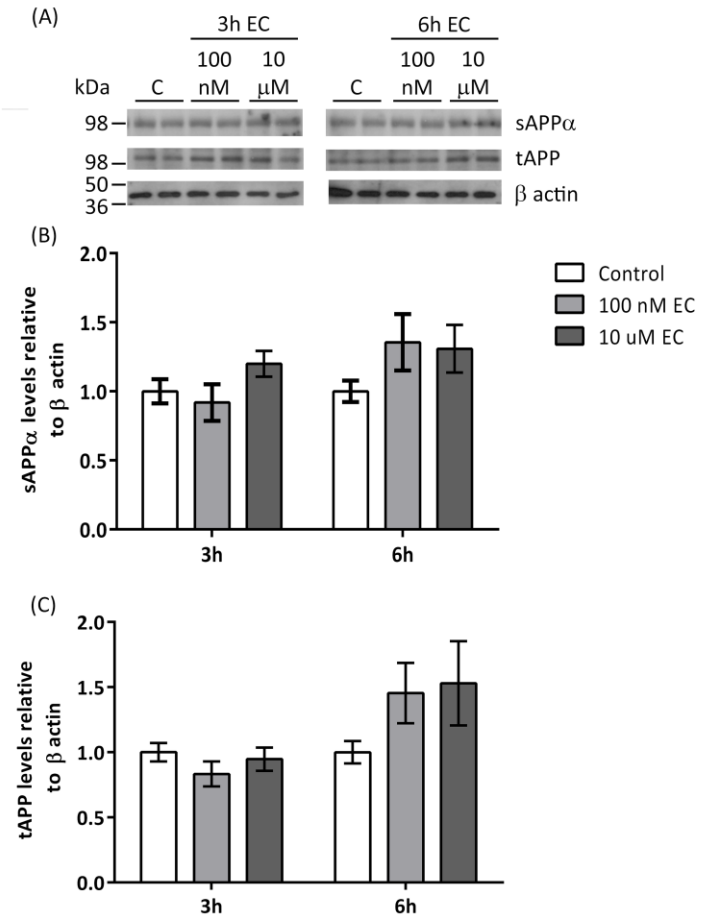



Figure 5.3: **(-) Epicatechin treatment does not affect released sAPPα levels in SH-SY5Y cells**

(A) SHSY5Y cells were treated with vehicle (C) or (-) epicatechin (100 nM and 10 µM) for 3h and 6h followed by immunoblotting for sAPPα (5G11), APP (22C11) or β actin. sAPPα levels were measured from conditioned media. $APP_{695}$ and β actin levels were measured from whole cell lysate. Levels of (B) sAPPα and (C) tAPP relative to β actin were analysed by ECL protein band densitometry using calibrated Image J software. Each point represents the mean ± S.E.M. Representative blots from three independent experiments.

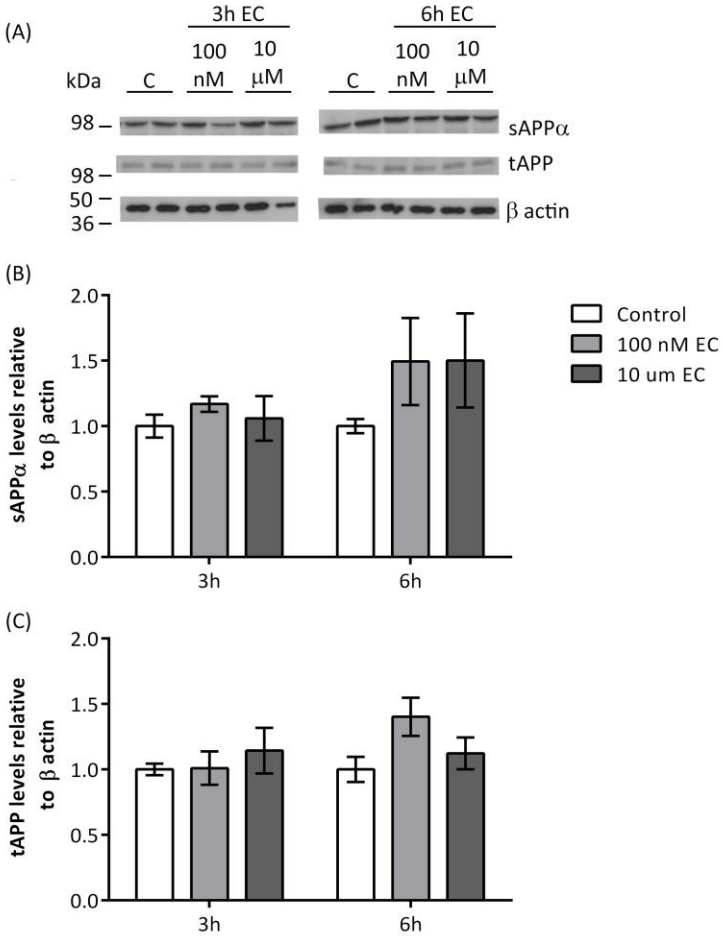



Figure 5.4: **(-) Epicatechin does not affect released sAPPα levels in HEK293T cells**

(A) HEK293T cells were treated with vehicle (C) or (-) epicatechin (100 nM and 10 μM) for 3h and 6h followed by immunoblotting for sAPPα (5G11), APP (22C11) or β actin. sAPPα levels were measured from conditioned media. $APP_{695}$ and β actin levels were measured from whole cell lysate. Levels of (B) sAPPα and (C) tAPP relative to β actin were analysed by ECL protein band densitometry using calibrated Image J software. Each point represents the mean ± S.E.M. Representative blots from three independent experiments.

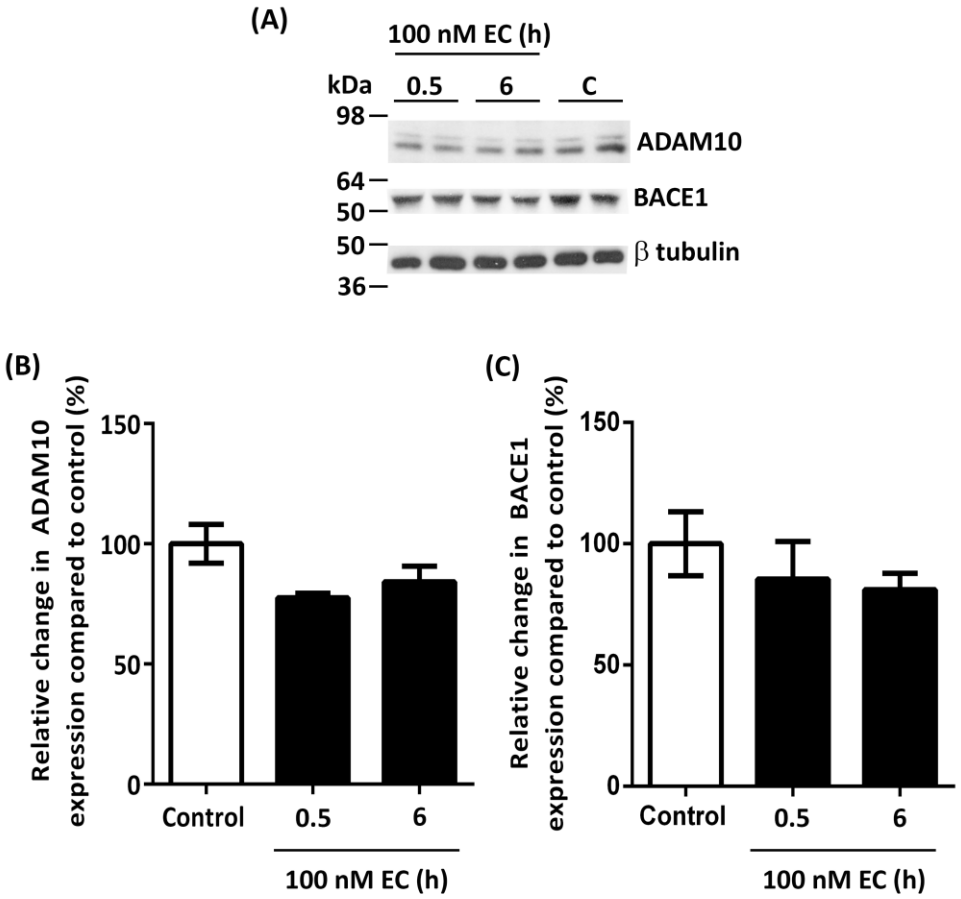



Figure 5.5: **(-) Epicatechin treatment does not affect α- or β-secretase levels in primary cortical neurons**

(A) 6 DIV primary C57/BL6 cortical neurons were treated with vehicle (C) or (-) epicatechin (100 nM) for 30 min or 6h followed by immunoblotting for ADAM10 (735-749), BACE1 (2C13) or β tubulin. Levels of (B) ADAM10 and (C) BACE1 relative to β tubulin were analysed by ECL protein band densitometry using calibrated Image J software. Each column is the mean ± S.E.M. of 4 independent treatments from 2 separate cultures.

### 5.2.1. (-) Epicatechin indirectly inhibits BACE1 activity

As it appeared that levels of BACE1 were not altered, the possibility that (-) epicatechin was acting at the level of BACE1 activity was investigated. To do this, a cell free, recombinant BACE1 assay was employed. Initial characterisation of the assay showed a direct, linear relationship between standard substrate concentration and fluorescence intensity (Figure 5.6(A)). To test the sensitivity of the assay, a concentration response utilising the β-secretase inhibitor BsI was performed. BsI (0.1-10 μM) was incubated with BACE1 for 6h and showed an $IC_{50}$ of 117 nM and maximal inhibition of 96.1% compared to control (Figure 5.6(B)). In order to validate the assay as a specific measure of BACE1 activity, two commercial β-secretase inhibitors, BsI and BiV, were incubated with BACE1 and showed 93% and 95% inhibition respectively compared to control. Incubation with the γ secretase inhibitor DAPT did not cause significant inhibition compared to control, supporting the specificity of the assay towards β-secretase modulators (Figure 5.6(C)). To investigate the effect of (-) epicatechin on BACE1 activity, (-) epicatechin (100 nM) was incubated with BACE1 for 6h and showed no effect. This suggested it was not acting as a direct catalytic inhibitor (Figure 5.6(C)). Alternatively, non-catalytic actions might be responsible for the inhibitory effects of epicatechin. Primary neurons were therefore treated with (-) epicatechin or epigallocatechin (0.01-10 μM) for 6h, lysates prepared and incubated with synthetic BACE1 substrate to measure endogenous BACE1 activity. (-) Epicatechin (100 nM) reduced BACE1 activity by 72% compared to BsI (Figure 5.6(D)). In comparison epigallocatechin caused maximum inhibition (86%) at 1μM. At higher concentrations of (-) epicatechin and epigallocatechin there was no inhibition of BACE1 activity (Figure 5.6(D)).

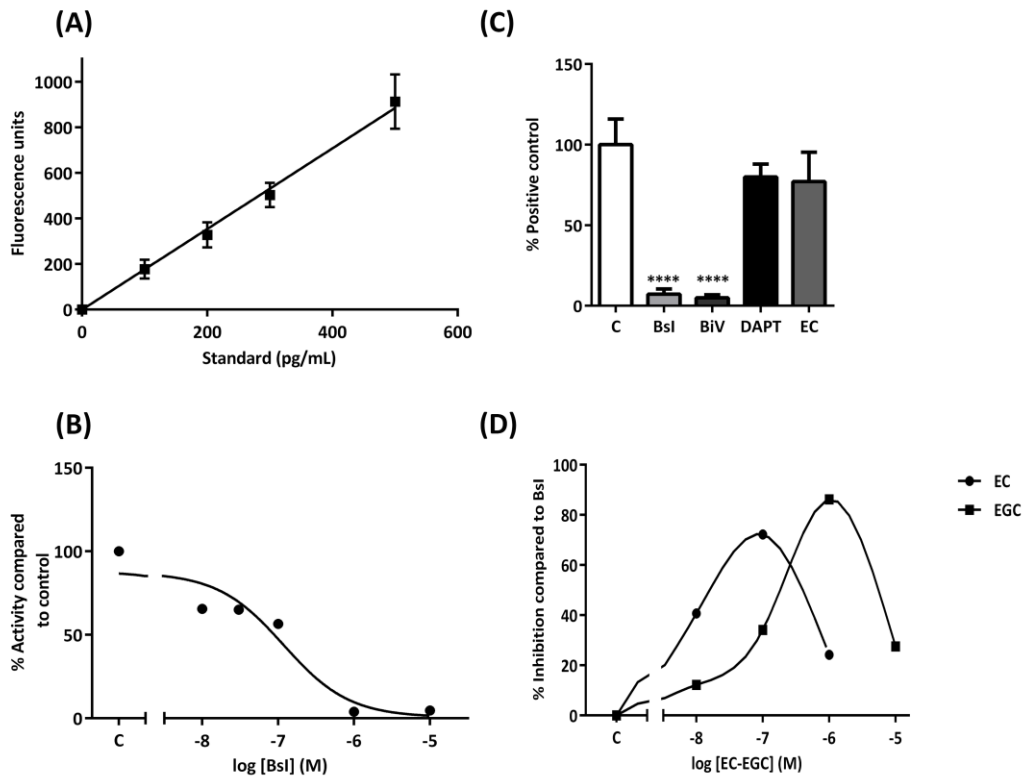

(A)
Fluorescence units
1000
800
600
400
200
0
0
200
400
600
Standard (pg/mL)
(C)
% Positive control
150
100
50
0
****
****
C
BsI
BiV
DAPT
EC
(B)
% Activity compared to control
150
100
50
0
C
-8
-7
-6
-5
log [BsI] (M)
(D)
% Inhibition compared to BsI
100
80
60
40
20
0
C
-8
-7
-6
-5
log [EC-EGC] (M)
EC
EGC

Figure 5.6: **(-) Epicatechin indirectly modulates BACE1 activity**

(A) Standard curve was calculated by serial dilution of 100 µM stock assay standard solution with fluorescent assay buffer. Levels of fluorescence at 405 nm were measured after 2h incubation. Curve was generated by subtraction of assay blank from all signal readings at 2h. All points are the mean ± S.E.M. of 4 independent experiments. (B) Assay components were incubated with vehicle or BsI (10 nM-10 µM) for 2h followed by measurement of fluorescence levels at 405 nm. Results expressed as % BACE1 activity compared to vehicle (control). (C) Assay components were incubated with vehicle (C), BsI (10 µM), BiV (10 µM), DAPT (10 µM) or EC (100 nM) for 2h followed by fluorescence measurement at 405 nm. Levels of fluorescence are expressed as % control levels. Each column is the mean ± S.E.M. of 3 independent experiments (n=3; ****$p<0.0001$ One way ANOVA with Bonferroni post-test). (D) Reduction of BACE1 activity in primary cell lysates treated with (-) epicatechin and epigallocatechin. Primary neuronal cultures were treated with 10 µM BsI (24h), (-) epicatechin (6h) or epigallocatechin (24h), the cells were then lysed and levels of BACE1 activity were measured following 2h incubation with synthetic peptide substrate. Data is represented as % inhibition compared to BsI (10 µM).

# 5.3. Discussion

This chapter aimed to address whether (-) epicatechin, was exerting its inhibitory effects on βγ-mediated APP processing and released Aβ through direct modulation of the proteolytic machinery responsible for APP metabolism. Biochemical analysis of primary neurons showed (-) epicatechin treatment did not alter $APP_{695}$ levels. Analysis of APP metabolites in primary neurons suggested that (-) epicatechin did not exert its effect through modulation of the non-amyloidogenic pathway, a finding supported by a lack of an (-) epicatechin mediated effect on ADAM10 expression. Investigation into the effects of (-) epicatechin on the amyloidogenic pathway suggested (-) epicatechin acts through indirect inhibition of BACE1 enzymatic activity.

## 5.3.1. (-) Epicatechin does not interfere with γ-secretase mediated notch cleavage

With respect to the development of (-) epicatechin for use in humans, an important consideration is the potential for inhibitory actions at γ-secretase. There have been a number of recent clinical trial failures for AD drugs due to off target effects at notch, leading to gastrointestinal and immune cell toxicity (Imbimbo et al., 2011, Doody et al., 2013). Assessment of (-) epicatechin effects on γ secretase is technically more challenging that the other secretase enzymes as it is a complex of proteins, therefore biochemical measurement of component protein levels does not necessarily reflect active complex. Furthermore, proteolytic products of γ secretase are either dependent on sheddase activity in the cases of Aβ and P3 or extremely technically challenging to detect in the case of free AICD. For these reasons, an assay measuring γ secretase activity at an alternative substrate, notch, was utilised which also allowed determination of off-target effects of (-) epicatechin. (-) Epicatechin did not inhibit notch cleavage at any concentration

tested suggesting that this is unlikely to be a significant hurdle to the development of (-) epicatechin as a potential treatment or prophylactic for AD. Lack of inhibition at notch also suggested that (-) epicatechin was not acting at the level of γ-secretase mediated cleavage of APP; however activity at the site of γ-secretase cleavage on APP could not be excluded.

### 5.3.2. $APP_{695}$ protein levels unaffected by (-) epicatechin treatment

$APP_{695}$ expression levels in primary neurons following (-) epicatechin treatment were not significantly changed. This suggests that the reduction in amyloidogenic processing seen following (-) epicatechin treatment is not due to lack of APP substrate but due to modulation of the proteolytic process. Previous studies investigating structurally related flavanol-mediated effects on APP processing have been shown to be independent of $APP_{695}$ levels, supporting an $APP_{695}$ holoprotein independent mechanism (Rezai-Zadeh, 2005, Wang et al., 2006).

### 5.3.3. (-) Epicatechin mediated effect on amyloidogenic APP processing is independent of up-regulation of sAPPα production.

Previous studies of a structurally similar catechin, EGCG, showed that it mediated up-regulation of sAPPα via enhanced ADAM10 maturation and mitigated Aβ levels in Swedish mutant APP overexpression cell lines, primary neurons and transgenic mice (Rezai-Zadeh, 2005, Obregon et al., 2006, Fernandez et al., 2010). EGCG formed part of the original screen and did not show any bioactivity, however lower concentrations of EGCG were tested in this study than that at which EGCG was shown to have potentiating effect on sAPPα. Therefore it was considered logical that (-) epicatechin could be a more potent molecule which acts through the same

mechanism as EGCG, but at lower concentrations. For this reason, levels of secreted sAPPα relative to β- tubulin were measured in primary neurons following (-) epicatechin treatment to investigate whether the same mechanism of action was responsible; however no changes in sAPPα levels were detected at any time point. A kinetic analysis was performed as although (-) epicatechin mediated effects in the APP-Gal4 reporter assay showed inhibitory activity at 6h, the dependence on expression of the gene reporter luciferase protein made extrapolation of this time-course difficult for measurement of endogenous APP modulations.

Firstly it is important to stress that the fact increased sAPPα levels were not found does not directly contradict the mechanistic results from the studies that investigated EGCG-mediated effects on APP. At the chemical level, structural differences between (-) epicatechin and EGCG, with (-) epicatechin lacking the gallate moiety on its B ring could explain the differences in bioactivity. There were also a number of experimental and biological differences between the studies. The main aim of this work was to identify mechanisms through which (-) epicatechin might exert its effects at physiological concentrations shown to be achievable in the mammalian brain following oral delivery. Studies showing EGCG as effective at increasing sAPPα levels utilised micromolar concentrations of EGCG, with maximal potentiation of sAPPα production at 20 μM, however concentrations of flavanols in the mammalian brain peak at 200-400 nM (van Praag et al., 2007, Wang et al., 2012). Therefore this mechanism of flavanol activation may be possible, however only at higher concentrations of flavanol. Although EGCG treatment in an *in vivo* mouse model of AD caused increased sAPPα levels and a concomitant increase in α-secretase activity, this was following IP injection, bypassing the need for intestinal absorption and allowing higher *in vivo* concentrations to be achieved (Rezai-Zadeh, 2005).

Further to this, work investigating potential cellular mechanisms of action for (-) epicatechin in this thesis has been carried out in WT primary neurons, whilst EGCG mediated mechanistic studies were predominantly carried out in the Swedish mutant APP-overexpressing neuron-like cell line N2A or primary neurons derived from Swedish mutant APP overexpressing transgenic mice (Tg2576). Therefore to

assess the cell type specificity of sAPPα production, 2 cell lines were employed. The SH-SY5Y cell line was chosen as a well-characterised *in vitro* model of neuronal function that constitutively expresses APP and releases considerable amounts of sAPPα (Levites et al., 2003), and HEK293T cells as a commonly used transformed cell line with high levels of non-amyloidogenic APP processing (Colombo et al., 2012). Both these cell lines when treated with (-) epicatechin showed no significant increases in sAPPα suggesting cell type or APP expression level does not impact on the underlying mechanism. It would still be of interest to investigate (-) epicatechin-mediated effects on sAPPα in transgenic primary cultures to fully assess this conclusion.

### 5.3.4. (-) Epicatechin acts as an indirect inhibitor of BACE1 activity independent of BACE1 expression

As the mechanism of action by which (-) epicatechin was acting in WT primary cortical neurons did not appear to be through up-regulation of the non-amyloidogenic pathway, the simplest potential mechanism for the observed (-) epicatechin effect was inhibition of BACE1.

Following (-) epicatechin treatment of primary neurons, sAPPβ levels did not change. This was in conflict with other results showing (-) epicatechin mediated Aβ reductions and inhibition of the APP-Gal4 assay (Chapter 4). Furthermore, although shown not to be direct catalytic inhibitors of BACE1, lysates from (-) epicatechin (6h) and epigallocatechin (24h) treated cells exhibited reduced endogenous BACE1 activity. This suggests an indirect inhibitory mechanism of action at BACE1 that is common to both catechin family members, although (-) epicatechin was a more potent inhibitor of endogenous BACE1 activity. To address the cause of reduced BACE1 activity, measurements of BACE1 expression levels were made. However, no changes in BACE1 protein expression were detected following (-) epicatechin treatment. It was therefore more likely that post-translational mechanisms were responsible. The precise mechanism is unclear but could involve actions at an

allosteric site, post-translational modification, or modulation of BACE1 localisation as recently reported for stigmasterol, a plant-derived phytosterol, which also reported no significant changes in BACE1 expression (Burg et al., 2013).

The lack of effect on sAPPβ levels following (-) epicatechin treatment whilst Aβ and amyloidogenic processing were reduced could be due to different sensitivities of the techniques used to measure the various APP metabolites. As a product of the dominant APP processing pathway in neurons, secreted sAPPβ levels are substantial in primary cultures, and due to its high stability, it accumulates as the neurons mature (Morales-Corraliza et al., 2009). The sensitivity of immunoblotting therefore may not be sufficient to identify small modulations by (-) epicatechin that were evident in measurements of Aβ and AICD-dependent signals.

Elucidation of an indirect mechanism of action for (-) epicatechin at the level of BACE1 means that the primary cellular target of (-) epicatechin is still to be identified. Several factors are known to affect BACE1 activity, including membrane cholesterol content, neuronal activity and the oxidative status of the cell.

Mild oxidative stress alters BACE1 subcellular compartmentalisation, increasing its co-localisation with APP and subsequently increasing BACE1 activity in neurons (Tan et al., 2013). Although the growing consensus is that flavonoids do not act as antioxidants in the brain, they have been shown to up-regulate antioxidant enzymes such as endothelial nitric oxide synthase (Mann et al., 2007). Therefore (-) epicatechin may reduce BACE1 activity through indirectly reducing the oxidative stress levels of the neuronal culture. BACE1 C-terminal domain phosphorylation has been heavily implicated in BACE1 cellular distribution and in particular C-Jun N-terminal kinase (JNK) activation has been shown to mediate oxidative stress-induced modulation of BACE1 (Tamagno et al., 2009). Interestingly, (-) epicatechin protects primary neurons from pro-oxidant insults through inhibition of JNK activation (Schroeter et al., 2001) suggesting (-) epicatechin could modulate BACE1 localisation through inhibition of this signalling pathway.

Alternatively, (-) epicatechin may be able to modulate BACE1 activity through signalling pathways associated with neuronal activity. Neuronal activity modulates

production and secretion of Aβ by modulating APP processing upstream of γ secretase cleavage (Kamenetz et al., 2003). Recent evidence suggests that neuronal activity stimulated by glycine or picrotoxin regulates co-localisation of APP and BACE1. Following activity induction, APP is directed to BACE1 containing endosomal compartments, leading to increased amyloidogenic processing. This co-localisation was shown to be clathrin-mediated endocytosis dependent (Das et al., 2013). In contrast, independent studies report that NMDAR- and AMPAR-dependent neuronal activity reduce Aβ production through a MAPK-dependent up-regulation of sAPPα production and concurrent suppression of amyloidogenic APP processing (Hoey et al., 2009, Verges et al., 2011, Hoey et al., 2013). These reports appear contradictory, however regulation of APP at the synapse has been shown to be dependent on the level of activity. In particular, NMDA-mediated stimulation of neurons is pro-amyloidogenic at low concentrations but anti-amyloidogenic at high concentrations (Verges et al., 2011). Both mechanisms are thought to be active simultaneously and it is the balance between them that determines the relative Aβ production level.

Stimulation of ERK activation and induction of GluA2 expression by (-) epicatechin (Schroeter et al., 2007) suggests it has the potential to regulate synaptic function. Through alteration of signalling pathways involved in maintaining the balance between pro- and anti-amyloidogenic APP processing at the synapse, (-) epicatechin may be able to indirectly modulate BACE1 activity. (-) Epicatechin improves spatial memory and promotes basal synaptic transmission and LTP, suggesting it can increase synaptic strength and thus levels of synaptic activity, which would lead to a shift in APP processing away from Aβ production (van Praag et al., 2007, Wang et al., 2012).

Flavonoids are proposed to have multi-modal activities (Maher et al., 2006) and so up-regulation of antioxidant enzymes and modulation of synaptic activity could be two complementary mechanisms acting to reduce Aβ levels following (-) epicatechin treatment. Without further evidence discounting one or both of these mechanisms they remain interesting hypotheses for future investigations.

In summary, (-) epicatechin-mediated reductions of βγ-secretase-mediated APP processing and Aβ levels have been shown to be independent of activity at the non-amyloidogenic pathway of APP processing. Investigation of (-) epicatechin mediated effects on amyloidogenic processing revealed an indirect inhibition of BACE1 activity that was not correlated with changes in BACE1 expression. Due to the strong spatial regulation of BACE1 activity, it is suggested that (-) epicatechin may exert its effect through modulation of cellular pathways involved in BACE1 trafficking and cellular localisation such as oxidative stress associated pathways and neuronal excitability.

# Chapter 6

## 6. Flavonoids differentially modulate ERK signalling in primary neurons

# 6.1. Introduction

## 6.1.1. ERK-mediated APP metabolism

In the brain, the ERK cascade plays a crucial role in synaptic plasticity and memory formation. High ERK activity in the hippocampus is associated with the best capacity for long-term memory formation (Eckel-Mahan et al., 2008). Inhibition of ERK activity causes memory deficits, specifically in memory retention (Hebert and Dash, 2002, Kelly et al., 2003). As well as the canonical role of ERK in activation of gene transcription through nuclear translocation and targeting of CREB and Elk to initiate gene transcription of genes such as *zif268* and *Arc* (Adams et al., 2000, Ying et al., 2002, Davis and Laroche, 2006), it also plays a crucial role at distal sites of the neuron. Here it regulates protein activity and localisation to dendritic and synaptic compartments. ERK-dependent cellular mechanisms modulate local protein synthesis at dendritic spines, such as modulation of calcium/calmodulin dependent kinase II (CaMKII) translation, a process required for LTP (Giovannini et al., 2001, Miller et al., 2002). They also regulate trafficking of extrasynaptic pools of AMPAR subunits to the synapse (Zhu et al., 2002, Kim et al., 2005b), and formation and stabilisation of dendritic spines (Goldin and Segal, 2003), essential structural changes associated with memory formation. ERK signalling is intimately linked with synaptic activity. Therefore the loss of synaptic function in AD, points to a potential link between ERK regulation of synaptic activity and APP processing.

APP processing and the production of Aβ can be regulated by the ERK-dependent mitogen-activated protein kinase (MAPK) signalling pathway in neurons (Hoey et al., 2009). Levels of activated ERK are elevated in the cerebral spinal fluid (CSF) and brains of AD patients (Perry et al., 1999, Klafki et al., 2009), possibly as a response to AD-related changes such as oxidative stress. This suggests that ERK signalling may be implicit in pathological changes associated with AD and therefore could act

as a novel therapeutic target. ERK regulation of synaptic activity highlights this as a potential mechanism of ERK involvement in AD.

Synaptic activity regulates Aβ generation (Kamenetz et al., 2003). Conflicting studies suggest synaptic activity can either increase or decrease neuronal Aβ production (Lesne et al., 2005, Hoey et al., 2009) although under physiological conditions activity-dependent suppression of Aβ is most likely to occur. NMDAR induced reduction of Aβ production is ERK dependent, suggesting ERK activity plays a key role in inhibition of Aβ levels both *in vitro* and *in vivo* (Kim et al., 2005b, Hoey et al., 2009, Verges et al., 2011, Kaufman et al., 2012, Wan et al., 2012). AMPAR activation also causes a reduction in Aβ production independent of NMDAR activity, which is at least partially ERK activity dependent (Hoey et al., 2013). Administration of MEK inhibitors, which lies upstream of ERK, increases Aβ interstitial fluid (ISF) levels suggesting that there is a physiological suppression of amyloidogenic processing by ERK signalling *in vivo* (Verges et al., 2011).

The ERK dependent suppression of amyloidogenic processing is not unique to glutamate-dependent stimulation of the pathway. Treatment of primary neurons with brain derived neurotrophic factor (BDNF) decreased $A\beta_{1\text{-}40}$ levels by approximately 40%, this was shown to be blocked by the MEK inhibitor UO126 (Rohe et al., 2009). Following activation, ERK is observed in both dendritic and somatic regions of neurons (Thomas and Huganir, 2004). In non-neuronal cells, translocation of activation ERK from the cytoplasm to the nucleus is a well-characterised phenomenon. However, in neurons nuclear translocation and transcriptional activation is unlikely to be the only functional outcome of ERK activation following stimulation at the synapse, which often causes ERK activity in subcellular compartments additional to the nucleus.. For short-term plasticity changes and rapid regulation of APP processing it is perhaps more likely that ERK acts on cytoplasmic target proteins, closer to synapses where activation of ERK was initiated (Thomas and Huganir, 2004).

### 6.1.2. Flavonoids as regulators of ERK activation

Accumulating evidence suggests that flavonoids interact selectively with MAPK signalling cascades. As already discussed, ERK activity is associated with cellular pathways thought to be responsible for learning and memory. Specific flavonoids including (-) epicatechin, fisetin and nobiletin and a flavonoid-rich pomegranate juice improve performance in behavioural tests of memory in mice (Hartman et al., 2006, Maher et al., 2006, Onozuka et al., 2008, Wang et al., 2012), with ERK signalling providing a probable mechanistic link.

A growing body of evidence suggests that flavonoids are able to modulate ERK activation in neurons. (-) Epicatechin and fisetin have been shown to enhance ERK-dependent activation of CREB, a pathway associated with LTP (Maher et al., 2006, Schroeter et al., 2007). Anti-apoptotic properties of some flavanones are mediated by ERK activation (Vauzour et al., 2007) and the flavone apigenin sustains EGF-stimulated ERK activation, correlating with downstream p90 ribosomal S6 kinase activation (Llorens et al., 2002). In contrast the flavonol quercetin potently inhibited ERK activation, leading to neuronal cell death. However, this effect was concentration dependent, with lower quercetin concentrations inducing increased CREB phosphorylation (Spencer et al., 2003). These differential signalling actions of flavonoids at varying concentrations are commonly observed, with low nanomolar concentrations typically stimulating ERK phosphorylation (Schroeter et al., 2007, Rainey-Smith et al., 2008). Collectively, these studies suggest that modulation of ERK activity is a property conserved across the flavonoid subfamilies and given that ERK regulates APP processing this represents a potential locus through which flavonoids might modulate Aβ production.

The aim of this chapter was to determine whether the lead flavonoids identified in the initial APP-Gal4 gene reporter assay, were modulators of ERK activity. Two approaches were adopted: biochemical analysis of active ERK using phosphorylation state-specific antibodies and a viral Egr1-luciferase reporter assay to measure MAPK dependent transcription.

# 6.2. Results

In Chapter 4 select flavonoids were found to decrease βγ-secretase mediated APP processing as measured by an APP-Gal4 luciferase gene reporter assay. It was also shown that treatment with (-) epicatechin and epigallocatechin, two of the flavonoids identified as inhibitory in the reporter assay, led to an indirect inhibition of BACE1 activity, independent of BACE1 expression levels. Under physiological conditions, ERK signalling has an inhibitory effect on amyloidogenic APP processing (Hoey et al., 2009, Verges et al., 2011). It was therefore investigated whether flavonoids identified as inhibitors of βγ-mediated APP processing were also modulators of the ERK signalling cascade.

## 6.2.1. (-) Epicatechin treatment causes a trend towards an increased pERK levels

Activation of ERK requires dual phosphorylation on tyrosine and threonine residues within the TEY motif of the catalytic domain (Seger et al., 1992, Roskoski, 2012). To investigate the effects of five lead flavonoids on ERK signalling the levels of active ERK were measured using a phosphorylation state specific antibody by western blot analysis. Flavonoid treatments (fisetin, pelargonidin chloride, sinensetin, (-) epicatechin or epigallocatechin) of 100 nM were again chosen as a physiologically relevant concentration achievable in the brain; 10 µM was investigated due to the biphasic activity of flavonoids reported at other targets. A short, 15 min stimulation was chosen based on previous reports of the kinetics of pERK activation in neurons. (-) Epicatechin induced the largest increase in ERK activation at 100 nM, resulting in an average increase of ~75% but with a high level of variance between experiments due to changes in basal levels of phosphorylated ERK(Figure 6.1). (-) Epicatechin was also the most effective flavonoid at 10 µM, causing a ~50% mean increase in pERK (Figure 6.2). Although there were trends towards increases in ERK activation

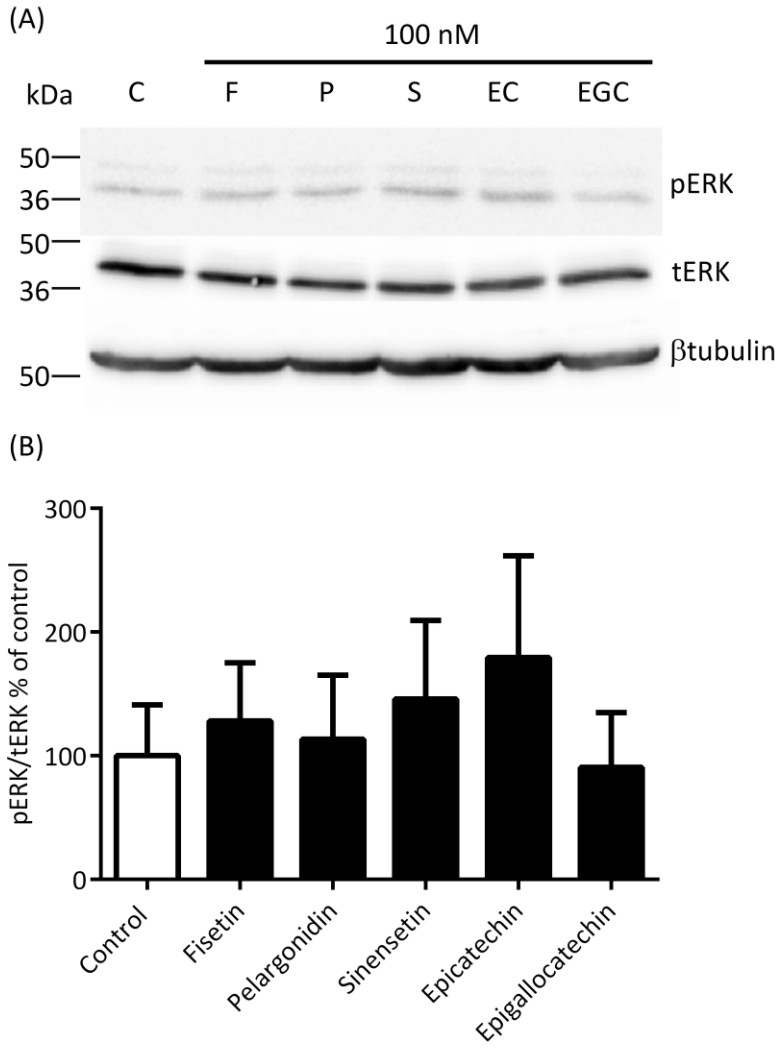



Figure 6.1: **Trend towards acute activation of ERK in primary neurons by 100 nM (-) epicatechin**

(A) 7 DIV primary cortical neurons were treated with vehicle (C), fisetin, pelargonidin, sinensetin, (-) epicatechin or epigallocatechin (100 nM) for 15 min followed by immunoblotting with p42/44 (pERK), ERK2 (tERK) and β tubulin antibodies. (B) pERK and tERK levels were analysed by ECL protein band densitometry using VilberLourmat imaging software. Levels of pERK expressed relative to tERK and transformed as a % of control. Each column represents the mean ± S.E.M. from 4 independent experiments.

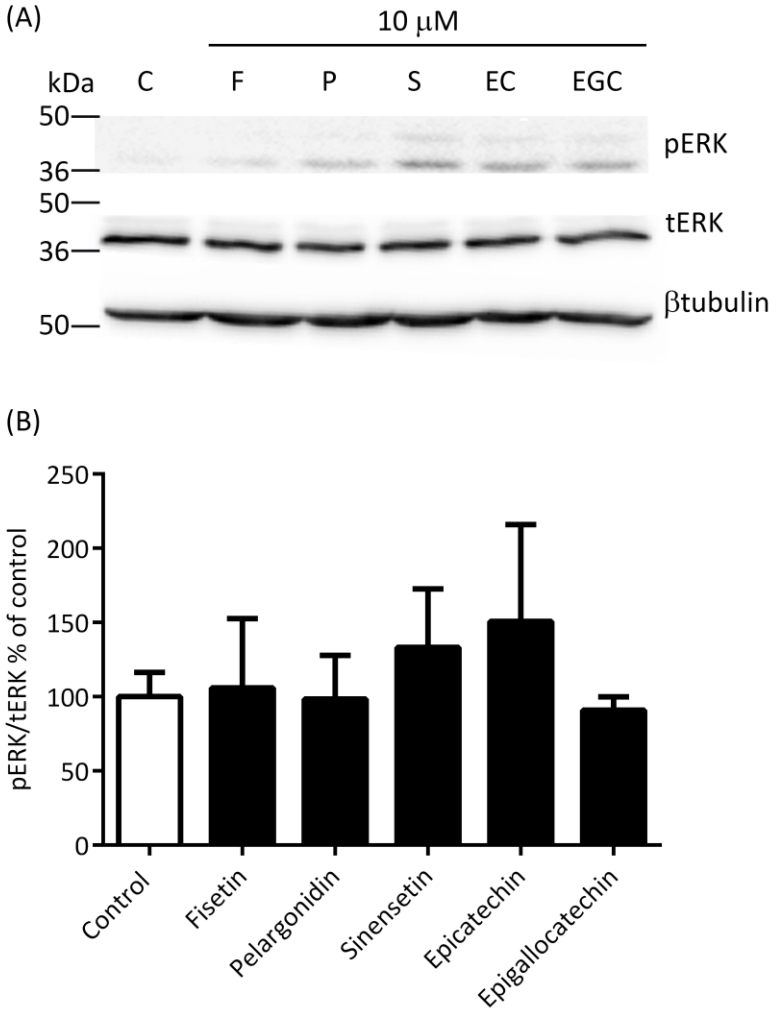



Figure 6.2: **Trend towards acute activation of ERK in primary neurons by 10 μM (-) epicatechin**

(A) 7 DIV primary cortical neurons were treated with vehicle (C), fisetin, pelargonidin, sinensetin, (-) epicatechin or epigallocatechin (10 μM) for 15 min followed by immunoblotting with p42/44 (pERK), ERK2 (tERK) and β tubulin antibodies. (B) pERK and tERK levels were analysed by ECL protein band densitometry using VilberLourmat imaging software. Levels of pERK expressed relative to tERK and transformed as a % of control. Each column represents the mean ± S.E.M. from 4 independent experiments.

following (-) epicatechin treatment, these did not reach statistical significance. The consistency in the level of pERK at both concentrations was seen for all 5 flavonoids, ranking (-) epicatechin > sinensetin > fisetin > pelargonidin > epigallocatechin at both concentrations (Figure 6.1 and 6.2).

### 6.2.2. BDNF stimulates MEK-dependent Egr-1 promoter-driven luciferase expression

The levels of pERK are known to correlate well with ERK activity but they do not indicate whether activity is spatially restricted within the dendrites or whether activated ERK possesses the potential to recruit transcription. In addition the kinetics of ERK activation and phosphatase-driven deactivation creates some hurdles to using this approach for flavonoid screening. Due to the levels of variability seen with this standard biochemical approach and a relative lack of sensitivity using immunoblotting, a different methodology was adopted utilising viral transduction of an Egr1-luciferase reporter to measure ERK dependent transcription. Primary neurons were transduced with the Ad5-Egr-1-luciferase reporter and then treated with BDNF (50 ng/mL) over 24h. BDNF evoked a maximal 5-fold induction of early growth response gene-1 product (Egr-1) mediated luciferase expression at 6h which was maintained up to 24h (Figure 6.3). This induction was completely blocked by co-treatment with two different MEK1/2 inhibitors, UO126 and PD184352 at all the time points tested (Figure 6.3).

### 6.2.3. NMDAR- and AMPAR-activation induce Egr-1 promoter-dependent luciferase expression

Glutamate receptors stimulate ERK activation, as measured by phosphorylated ERK levels (Sweatt, 2004). However, what is much less clear is whether this activation results in transcriptional activity at ERK target genes. To address this, primary neurons were transduced with an Ad5-Egr-1-luciferase reporter and then treated.

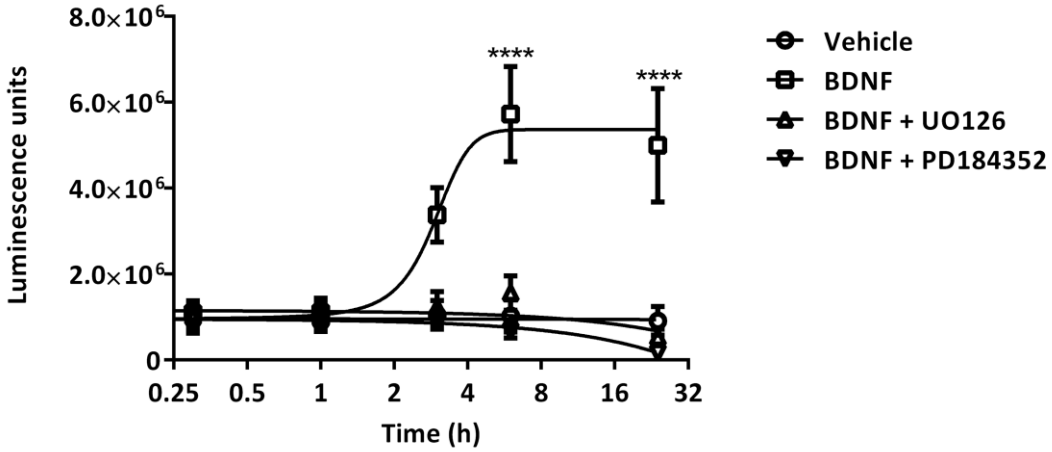



Figure 6.3: **BDNF stimulates ERK dependent transcriptional activity in primary neurons**

6 DIV primary cortical neurons were transduced with $5x10^5$ pfu/mL Ad5-Egr-1-luciferase and treated with vehicle, BDNF (50 ng/mL), BDNF and UO126 (10 µM) or BDNF and PD184352 (2 µM) over 24h. Luciferase activity assays were performed 24h post treatment for quantification of luciferase expression. Each point is mean ± S.E.M. of 3 independent experiments (n=3; **** $p<0.0001$ compared to vehicle, two way ANOVA with Bonferroni post-test).

with NMDA or AMPA over 24h. NMDA and AMPA both induced time-dependent increases in Egr-1 mediated luciferase expression compared to control

The NMDA and AMPA effects were of similar magnitude and timecourse. NMDA reached a maximal induction of 291% compared to control at 6h whilst AMPA reached maximal induction of 209 % compared to control at 6h (Figure 6.4(A)).

### 6.2.4. NMDA-evoked Egr-1 promoter-driven luciferase expression is MEK dependent

Next it was necessary to determine whether the glutamate receptor agonist promotion of Egr-1 promoter-driven luciferase expression was mediated by MEK-dependent activation of ERK. Primary neurons transduced with the Ad5-Egr1-luciferase reporter were treated with NMDA in the presence or absence of UO126 or PD184352. The time-dependent stimulation of Egr-1 promoter driven luciferase expression by NMDA was blocked by both UO126 and PD184352 (Figure 6.4(B)). Furthermore, by 24h both MEK inhibitors has caused a substantial reduction in the levels of Egr-1 mediated luciferase expression compared with control, with maximal reduction of 19% (UO126) and 18% (PD184352) of control (Figure 6.4(B)).

### 6.2.5. Quercetin co-treatment suggests a flavonol-dependent reduction in NMDA-induction of Egr-1 promoter-driven luciferase expression

The flavonol quercetin formed the structural basis of a potent lipid kinase inhibitor LY294002, inhibiting PI3K with an $IC_{50}$ of 1.4 µM (Vlahos et al., 1994). Due to its known kinase inhibitor properties it was tested for modulatory effects on Egr-1-promoter dependent luciferase expression. Treatment with NMDA for 6h increased average Egr-1 promoter dependent luciferase expression by 50% compared to control (Figure 6.5), although this was not significant due a lack of experimental

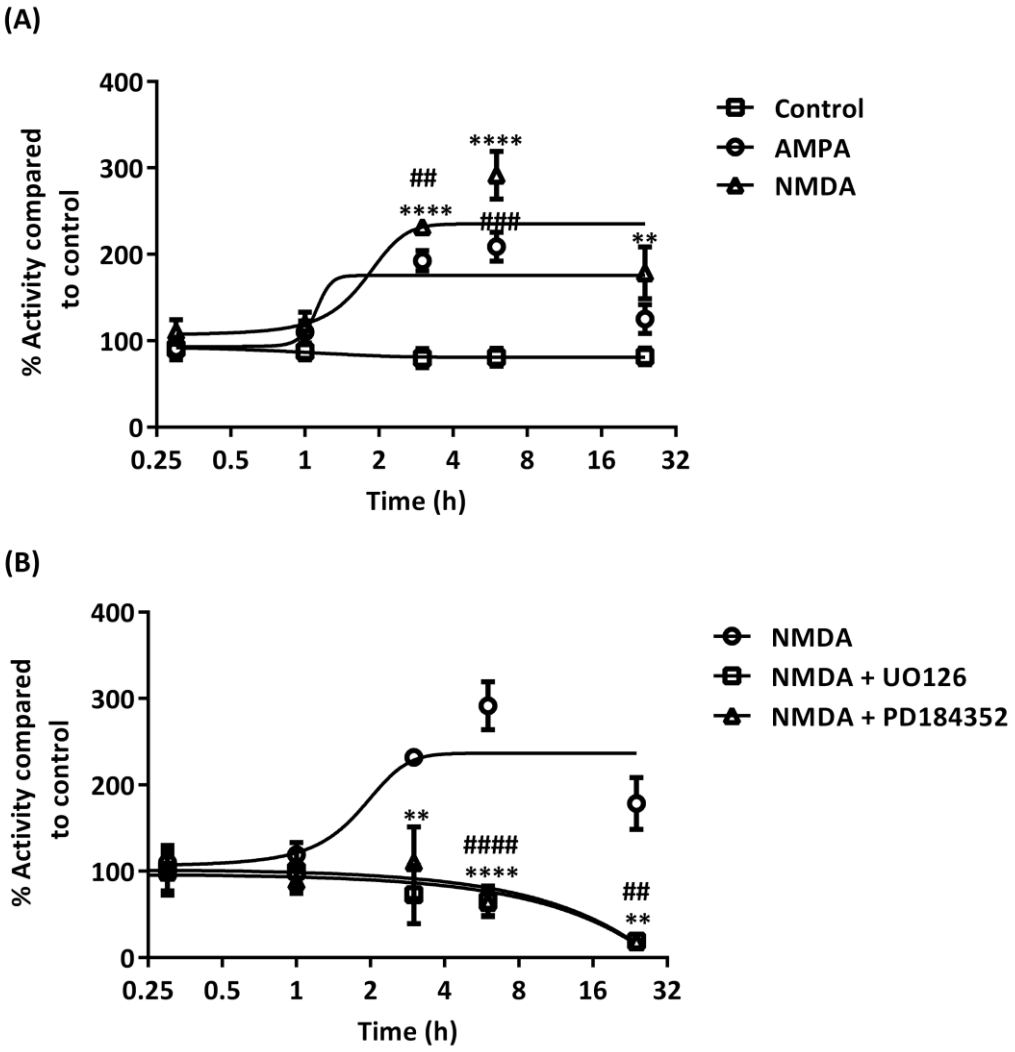



Figure 6.4: **NMDA and AMPA stimulated ERK dependent transcriptional activity in primary neurons**

6 DIV primary cortical neurons were transduced with $5x10^5$ pfu/mL Ad5-Egr-1-luciferase and treated with (A) vehicle (Control), NMDA (50 µM) or AMPA (50 µM) and (B) NMDA (50 µM), NMDA and UO126 (10 µM) or NMDA and PD184352 (2 µm) over 24h. Luciferase activity assays were performed 24h post treatment for quantification of luciferase expression. Each point is mean ± S.E.M. of 3 independent experiments (n=3) (A) NMDA** p< 0.01 **** p<0.0001, AMPA ## P<0.01 ### P< 0.001 compared to control, two way ANOVA with Bonferroni post-test). (B) NMDA + UO126 ** P< 0.01 **** P< 0.0001, NMDA +PD184352 ## p<0.01 #### p<0.0001 compared to NMDA, two way ANOVA with Bonferroni post-test).

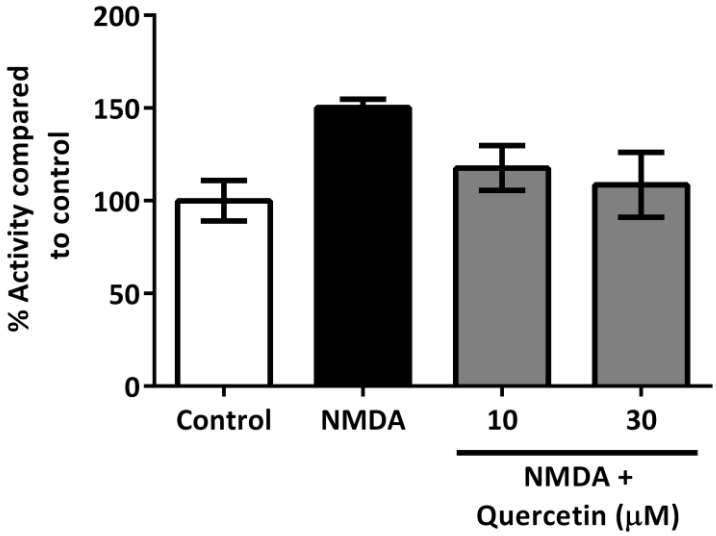



Figure 6.5: **Co-treatment with quercetin suggested that NMDA-mediated stimulation of ERK-dependent transcriptional activity is reduced by the flavonol in primary neurons**

6 DIV primary cortical neurons were transduced with $5x10^5$ pfu/mL Ad5-Egr-1-luciferase and subsequently treated with vehicle (Control), NMDA (50 μM), NMDA and quercetin (10 μM) or NMDA and quercetin (30 μM) for 6h. Luciferase activity assays were performed 24h post transduction for quantification of luciferase expression. Each point is mean ± S.E.M. of 2 independent experiments.

repeats. Co-treatment of transduced primary neurons with NMDA and quercetin (10 and 30 µM) for 6h showed a trend towards reduced luciferase expression to 117 and 108% of control respectively (Figure 6.5) suggesting inhibitory activity within the ERK cascade.

### 6.2.6. Fisetin treatment shows trend towards reduced basal Egr-1 promoter-induced luciferase expression levels

Due to positive indications from the biochemical analysis of lead flavonoid-mediated induction of ERK phosphorylation and the modulatory effects of quercetin in the Egr-1 luciferase assay, transduced primary neurons were treated with 10 and 30 µM of each flavonoid for 6h. No flavonoid showed potentiation of Egr-1 dependent luciferase expression. Contrary to the western analysis of phosphorylation ERK levels, fisetin showed a concentration dependent trend towards inhibition of Egr-1 dependent luciferase expression causing maximal inhibition of 43% of control, although this inhibitory trend was not significant (Figure 6.6).

### 6.2.7. Fisetin and (-) epicatechin show inhibitory and potentiating trends respectively on NMDA mediated Egr-1 promoter-driven luciferase expression

It has been demonstrated that NMDA mediated ERK activation leads to changes in APP processing, resulting in reduced Aβ levels. To investigate whether the lead flavonoids could modulate this NMDA-mediated ERK activation, each flavonoid in combination with NMDA was added to primary neurons. Levels of Egr-1-dependent luciferase expression were measured 6h later. In agreement with the effect of fisetin on basal Egr-1 promoter-dependent luciferase expression (Figure 6.6), fisetin

showed an inhibitory trend towards reducing NMDA-stimulated Egr-1 promoter driven luciferase expression.(Figure 6.7). In contrast to the basal Egr-1 luciferase expression where (-) epicatechin did not evoke any change, (-) epicatechin when co-treated with NMDA showed a modest trend towards a concentration dependent potentiation of Egr-1 promoter driven luciferase expression. (-) Epicatechin enhanced Egr-1 promoter dependent luciferase expression by 209% (30 μM) compared to NMDA stimulation alone, however due to low power, this trend was not significant (Figure 6.7).

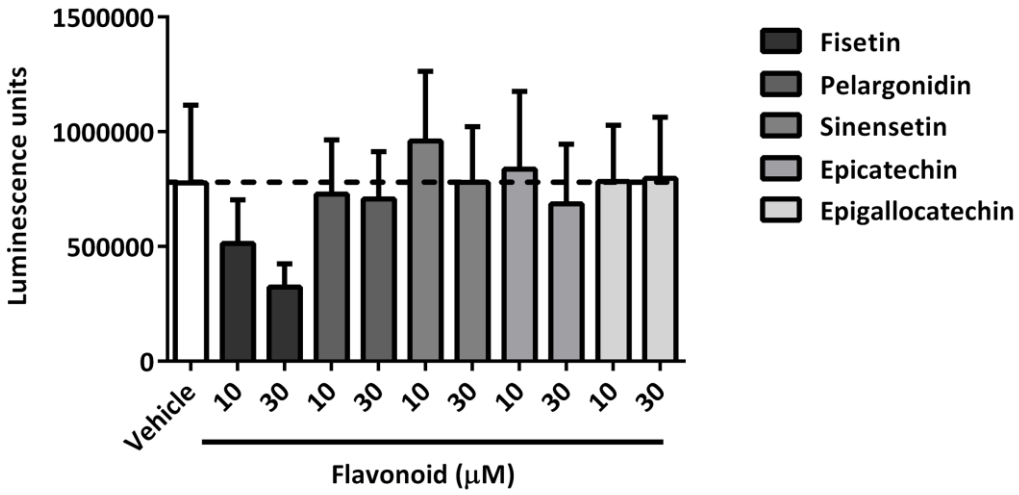



Figure 6.6: **Flavonol fisetin reduced basal ERK dependent transcriptional activity in primary neurons**

6 DIV primary cortical neurons were transduced with $5x10^5$ pfu/mL Ad5-Egr-1-luciferase and subsequently treated with vehicle, fisetin, pelargonidin, sinensetin, (-) epicatechin or epigallocatechin (10 and 30 μM) for 6h. Luciferase activity assays were performed 24h post transduction for quantification of luciferase expression. Each point is mean ± S.E.M. of 3 independent experiments.

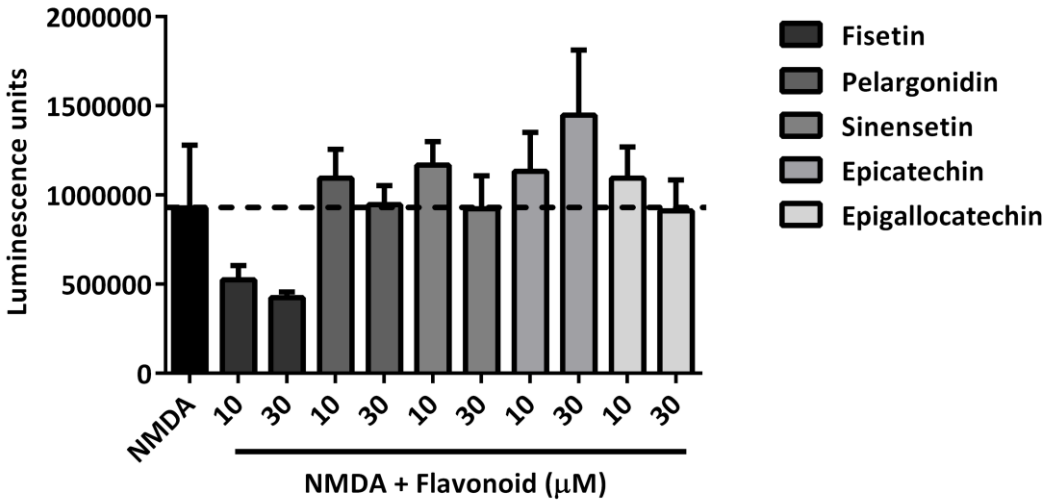



Figure 6.7: **Fisetin and (-) epicatechin showed inhibitory and potentiating trends towards NMDA-mediated stimulation of ERK transcriptional activity**

6 DIV primary cortical neurons were transduced with $5x10^5$ pfu/mL Ad5-Egr-1-luciferase and subsequently treated with NMDA (50 μM), or NMDA in combination with fisetin, pelargonidin, sinensetin, (-) epicatechin or epigallocatechin (10 and 30 μM) for 6h. Luciferase activity assays were performed 24h post transduction for quantification of luciferase expression. Each point is mean ± S.E.M. of 3 independent experiments.

# 6.3. Discussion

Up to this point, the focus of this thesis has been on flavonoid-mediated effects of APP processing and mechanisms through which they could be having a direct impact upon it. Failure to identify a direct mechanism for flavonoid regulation of APP processing, focussed attention on signalling pathways through which flavonoids may be able to exert their inhibitory actions on amyloidogenic processing. Flavonoid modulation of the MAPK signalling cascade, in particular ERK signalling has been reported previously and therefore an investigation into the activation of ERK activity by flavonoids identified in the APP-Gal4 screen was conducted. Measurement of activated ERK following acute treatment with lead flavonoids identified (-) epicatechin as a potent activator of ERK activity. (-) Epicatechin also potentiated NMDA-mediated up-regulation of ERK transcriptional activity, suggesting (-) epicatechin treatment leads to transcriptional regulation of targets. Further analysis showed the flavonol fisetin to be an inhibitor of ERK-dependent transcriptional activity, although the precise mechanism remains unclear as no change in active ERK1/2 was observed.

## 6.3.1. pERK blots suggest (-) epicatechin may up-regulate ERK signalling in primary neurons

Comparison of fisetin, pelargonidin, sinensetin, (-) epicatechin and epigallocatechin-mediated modulation of ERK activation, did not identify any significant changes in pERK levels however showed differences in trends of activity of the flavonoids. (-) Epicatechin treatment exhibited a clear trend towards increased pERK levels at both concentrations tested. It is likely that variability in control levels of ERK phosphorylation in the primary cortical cultures altered the effect size of (-) epicatechin treatment across experiments. Sinensetin and fisetin also displayed trends towards increased ERK activation, however these were not as strong as that

of (-) epicatechin. Pelargonidin and epigallocatechin had no effect on ERK activity at either concentration. (-) Epicatechin mediated- activation of ERK has been demonstrated previously in primary neurons (Schroeter et al., 2007), supporting this observation.

### 6.3.2. Egr-1 luciferase assay is a measure of ERK transcriptional activity in primary neurons

To investigate whether the lead flavonoids mediated ERK-dependent transcriptional activation, an Egr-1 luciferase viral assay was utilised. Activation of ERK and its translocation from the cytoplasm to the nucleus is necessary for the expression of many immediate early gene products, including Egr-1. A viral construct with a luciferase gene under the control of the Egr-1 promoter therefore can be utilised as a sensitive transcriptional readout for ERK activation (Caunt et al., 2008).

Biochemical and regulatory properties of post-mitotic CNS neurons are distinct from PNS neurons and non-neuronal cell types. As the Egr-1 luciferase assay had previously only been used in non-neuronal cell types, it was crucial to validate Egr-1 luciferase assay as a valid method for measurement of ERK-dependent transcriptional activity in primary neurons. BDNF is the most widely distributed neurotrophin in the brain and via its receptor TrkB has been shown to promote neuronal cell survival (Lu and Martinowich, 2008). These survival-promoting properties are elicited by activation of the ERK intracellular pathway both *in vitro* and *in vivo* and can be blocked with specific ERK inhibitors (Hetman et al., 1999, Han and Holtzman, 2000). As a well characterised activator of ERK in neuronal cells, BDNF stimulation of Egr-1 dependent luciferase expression was measured over a time course of 24h and showed a significant induction of ERK dependent transcriptional activity from 6h which was maintained to 24h. This effect was completely blocked by two structurally distinct MEK inhibitors UO126 and PD184352. This confirmed that the Egr-1 luciferase assay was a measure of ERK transcriptional activity in primary neurons.

### 6.3.3. NMDA- and AMPA-induced glutamatergic signalling causes ERK-dependent transcriptional activation in primary neurons

Following confirmation that the assay was sensitive to BDNF, the effects of ionotropic glutamate receptor agonists NMDA and AMPA were investigated. Under physiological conditions, glutamate binding to post-synaptic AMPAR leads to depolarisation of the post-synaptic membrane, this allows activation of NMDAR and an influx of $Ca^{2+}$ into the post-synaptic cell. This increase in intracellular $Ca^{2+}$ concentration triggers a series of intracellular signalling cascades (Bloodgood and Sabatini, 2007). Excitatory glutamatergic signalling has been demonstrated to activate ERK as part of a mechanism heavily implicated in LTP and synaptic plasticity (Thomas and Huganir, 2004). Of particular interest to this project, NMDA-mediated regulation of APP processing has also been shown to be ERK dependent and results also suggest a role for ERK activation in AMPA-mediated regulation of APP (Hoey et al., 2009, Verges et al., 2011, Hoey et al., 2013). Despite activation of ERK being conclusively demonstrated downstream of glutamatergic signalling, it remains unclear whether this is through ERK activity that mediated activation of transcription or through ERK modulation of cytoplasmic targets, or perhaps most interesting-both. NMDA treatment of transduced neurons resulted in a time dependent increase in ERK dependent transcriptional activity, reaching maximal activity at 6h, which was maintained to 24h. AMPA treatment of transduced neurons also resulted in a time dependent increase in ERK dependent transcription activity, reaching maximal activity at 3h and maintained over 24h. This provides direct evidence for ERK-driven transcriptional activity following glutamate receptor stimulation and supports previous evidence showing activation of nuclear transcription factors such as CREB (Impey et al., 1998, Roberson et al., 1999) and expression of immediate early genes such as *zif268* following glutamate receptor stimulation (Ying et al., 2002).

Blockade of ERK activation by MEK inhibitors UO126 and PD184352 blocked NMDA-mediated ERK-driven transcriptional activity, confirming that the NMDA mediated effects on Egr-1 dependent luciferase expression were ERK signalling dependent. Chronic inhibition by both UO126 and PD184352 caused ERK transcriptional activity to drop to 20% of control. This could be an indication of reduced cell viability following chronic ERK inhibition coupled with NMDA activation or that basal ERK activity was high in these cultures.

### 6.3.4. Fisetin treatment shows inhibitory trend on ERK-dependent transcriptional activity

It was next investigated whether transcriptional up-regulation of ERK could be a potential mechanism of action by flavonoids identified as inhibitors in the initial APP-Gal4 screen. Nanomolar concentrations of all flavonoids were ineffective at inducing ERK-driven transcriptional activity, however following micromolar flavonoid treatment over 24h, results suggested that the flavonol fisetin may down-regulate ERK transcriptional activity in a concentration dependent manner. Fisetin has previously been demonstrated to have activity at ERK, promoting differentiation of PC12 cells via activation of the ERK cascade (Sagara et al., 2004) and inducing ERK dependent CREB phosphorylation in primary rat hippocampal slices (Maher et al., 2006). Inhibition of ERK-driven transcriptional activity is difficult to correlate with these studies; however ERK signalling pathways are regulated differently in neuronal cell types compared to non-neuronal cell types which may explain these opposing roles for fisetin. Induction of CREB phosphorylation is highly correlated with transcriptional activation of ERK-related genes; however regulation of CREB as the rate-limiting molecular switch of transcription remains unclear. It therefore may be possible that the two observations of fisetin-mediated ERK-dependent CREB activation and suspected ERK transcriptional down-regulation are not mutually exclusive.

It must also be considered that fisetin may be neurotoxic to primary neurons and this, rather than a specific inhibition of ERK-driven transcriptional activity is causing the inhibitory trend Egr-1 promoter-dependent luciferase expression. However, fisetin has previously been shown to possess neuroprotective properties at micromolar concentrations in an *in vitro* model of stroke, and following $H_2O_2$ administration (Dajas et al., 2003, Maher et al., 2007). These reports question the probability of fisetin acting as a neurotoxic agent in primary neurons, thus supporting a more specific activity for fisetin at the level of the ERK signalling pathway.

No modulation of ERK phosphorylation was observed following fisetin treatment of primary neurons, however a strong trend towards ERK dependent transcriptional activity down-regulation following fisetin treatment was observed. Flavonoid-mediated changes in levels of ERK phosphorylation were measured at 100 nM and 10 µM whilst ERK transcriptional activity changes by fisetin were measured at 10 - 30 µM with no effect observed at 100 nM. This concentration-dependent paradox could be due to, or the cause of the biphasic activity seen for fisetin in the APP-Gal4 reporter assay. It would therefore be interesting to investigate the ERK dependence of the concentration dependent, biphasic activity of fisetin in primary neurons as it may elucidate a mechanistic explanation for the concentration-dependent activity of this flavonol.

### 6.3.5. Fisetin and (-) epicatechin show opposing trends on NMDA-mediated ERK transcriptional activity in primary neurons.

Select flavonoids, as shown in Chapter 3, can reduce levels of amyloidogenic processing and excitatory glutamatergic signalling also leads to ERK dependent reductions in Aβ levels *in vitro* and *in vivo* (Hoey et al., 2009, Verges et al., 2011). Investigation of flavonoid modulation of NMDA-mediated ERK-driven

transcriptional activation identified fisetin and (-) epicatechin as flavanols with potential activity at NMDA mediated ERK transcriptional activity.

Fisetin showed a similar inhibitory trend on NMDA-mediated ERK driven transcriptional activity as that seen on basal ERK activity, suggesting a target common to both mechanisms. Classical neuronal stimulation of ERK is caused by increased Ras-GTP levels in response to extracellular stimuli such as neurotrophins. Neuronal activation by membrane depolarisation and subsequent excitatory glutamatergic signalling requires increased intracellular $Ca^{2+}$ concentrations for activation of Ras-GTP (Fiore et al., 1993, Rosen et al., 1994) (Figure 6.8). These divergent initial mechanisms of activation suggest that potential cellular targets of fisetin may lie downstream of Ras in the ERK pathway or a modulator of one of the downstream effectors.

(-) Epicatechin caused a concentration dependent potentiation trend in ERK driven transcriptional activation when co-administered with NMDA. As (-) epicatechin exhibited no stimulatory effect when delivered alone, this suggests that (-) epicatechin may act to potentiate the NMDA-mediated increase in ERK activation. Further experiments to confirm the potentiating action of (-) epicatechin and studying the effect of this in combination with NMDA antagonists such as MK801 would allow further elucidation of this potential mechanism.

This trend towards (-) epicatechin potentiation of NMDA-mediated ERK driven transcriptional activity supports findings that (-) epicatechin enhances learning and memory in mice (van Praag et al., 2007). Synaptic plasticity is thought to underlie complex behaviours such as learning and memory. LTP is one of the best-characterised forms of synaptic plasticity and is therefore one of the most promising candidates for the cellular mechanism behind the behaviours. NMDAR-mediated-ERK activation is required for cortical LTP, therefore (-) epicatechin potentiation of this pathway is likely to enhance LTP and subsequent pathways leading to improvement in learning and memory described (van Praag et al., 2007, Wang et al., 2012).

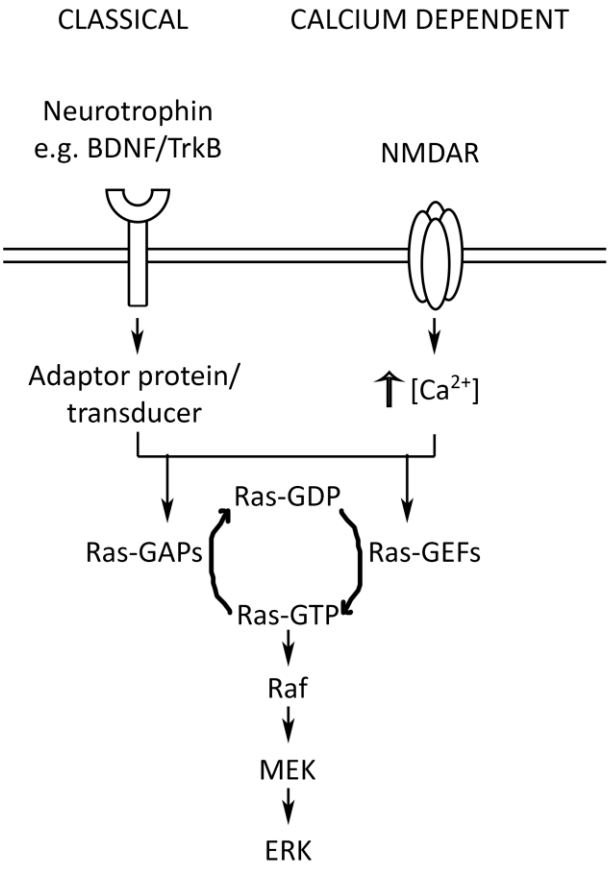



Figure 6.8: **Activation of ERK signalling in neuronal cells**

Classical activation of the ERK pathway by neurotrophins through receptor tyrosine kinases, activation of adaptor proteins and increased activity of guanyl nucleotide exchange factors (GEFs) triggers increased levels of Ras-GTP. ERK activation in response to glutamatergic signalling is different in that activation of Ras is intracellular $Ca^{2+}$ concentration dependent, where Ca2+ influx through NMDAR triggers Ras-GTP levels to increase. Downstream of Ras activation both pathways converge, with Ras activating Raf, mitogen activated protein kinase (MAPK)/ERK kinase (MEK) and ERK. Following its activation ERK can phosphorylate both cytoplasmic and nuclear targets.

In summary, flavonoid-mediated effects on ERK activation were concentration dependent and different flavonoids had contrasting effects at the same concentrations. This suggests that, at least at the level of flavonoid modulation of ERK activity, there is not a common mechanism across the flavonoid families and as these clear trends did not reach significance that ERK activation by flavonoids is possibly modulatory. (-) Epicatechin has been the only flavonoid to consistently show activity across all measures of ERK activity. Whether these effects are in addition to or are associated with the inhibition of amyloidogenic processing and BACE1 activity down-regulation is not clear. It has been proposed that flavonoids are capable of modulating multiple pathways associated with maintenance of brain function. Thus searching for a single mechanism that is responsible for all flavonoid mediated effects may not be the ideal approach for the study of these neuroprotective, antioxidant, and Aβ pathology ameliorating compounds.

# Chapter 7

## 7. General Discussion

# 7.1. Discussion

This thesis set out to address whether flavonoids were modulators of APP processing in primary neurons. An APP-Gal4 gene reporter assay was characterised and manipulated to provide a tractable system for the identification of novel modulators of APP processing in primary neurons. Using this system, five flavonoids were identified that inhibited amyloidogenic processing: fisetin, pelargonidin, sinensetin, (-) epicatechin and epigallocatechin. Investigations into (-) epicatechin-mediated inhibition of APP supported indirect inhibition of BACE1 activity as the mechanism of action. Interestingly, study of the lead flavonoids' effects on ERK signalling showed that (-) epicatechin may enhance ERK activation and activity-dependent ERK-driven transcriptional activity in primary neurons. In contrast, another lead flavonoid, fisetin, showed a trend towards suppression of ERK-dependent transcriptional activity. These data suggest that individual flavonoids do indeed modulate APP processing, and that they may regulate the ERK signalling cascade, likely through multiple cellular targets.

Through modification of the APP-Gal4 luciferase gene reporter assay, a highly sensitive, tractable assay system has been established which allows novel compounds to be tested for activity at APP processing. This can be conducted under physiological, as well as environmental and genetically-induced AD-associated conditions. The APP-Gal4 assay allows assessment of compounds of interest prior to appearance of AD pathology, allowing preventative compounds to be evaluated. The assay has been characterised as a measure of βγ-secretase mediated processing, greatly enhancing its utility, however the underlying cellular mechanism for this preference is unclear. It is therefore not possible to draw mechanistic conclusions about modulatory compounds identified. This is a significant limitation of the assay as mechanistic understanding of compound inhibition could provide critical new insight into regulation of APP processing.

Due to the post-mitotic nature of primary neurons, transfection efficiency is low compared to non-neuronal cell types. This meant that levels of the transfected APP protein were too low to be detected by traditional biochemical techniques. This meant that direct corroboration of assay hits, through APP metabolite measurements for example, could not be conducted. Generation of viral vectors for expression of APP-Gal4 would enhance the power of this assay as the number of cells expressing the exogenous APP-Gal4 would be greatly increased. Parallel measurements of APP processing modulation could then be performed, gaining mechanistic insight into the changes observed in the assay.

APP metabolism is central to the pathogenesis of AD. Rare mutations, either in the APP gene surrounding the secretase cleavage sites, or in the PS genes affecting γ-secretase cleavage of APP, cause autosomal dominant inheritance of AD (Larner, 2013, Tanzi, 2013). A major pathological hallmark of the disease is the presence of extracellular amyloid plaques, whose main constituent is Aβ, the proteolytic product of amyloidogenic processing of APP (Selkoe and Podlisny, 2002). Despite continuing controversy, soluble Aβ oligomers present as the strongest candidates for the neurotoxic species that initiates AD pathogenesis (Walsh et al., 2002, Lesné et al., 2006, Li et al., 2009). In contrast, a product of non-amyloidogenic processing, sAPPα, is neuroprotective and is suggested to be crucial for LTP (Gralle and Ferreira, 2007, Taylor et al., 2008). Inverse coupling of α- and β-secretase activities generates equilibrium between cellular levels of amyloidogenic and non-amyloidogenic APP processing in neuronal cells (Postina et al., 2004, Kuhn et al., 2010, Colombo et al., 2012). This dynamic interaction between the alternative pathways of APP processing provides an increased number of cellular targets for the treatment of AD through modulation of APP processing. Up-regulation of the non-amyloidogenic pathway as well as down-regulation of the amyloidogenic pathway represents two viable targets for development of novel therapeutics.

Epidemiological studies suggest that consumption of flavonoid rich foods is associated with prevention or delay of onset of AD. The Kame, Paquid, and Three City cohort longitudinal studies of aged subjects all found reduced AD incidence in populations with higher flavonoid intake (Commenges et al., 2000, Dai et al., 2006,

Barberger-Gateau et al., 2007, Letenneur et al., 2007). *In vivo* studies, consistently report flavonoid-mediated inhibition of Aβ levels associated with improvements in behavioural tests of memory (Hartman et al., 2006, Rezai-Zadeh et al., 2008, Mori et al., 2012, Wang et al., 2012). These results suggest that flavonoid-mediated reductions in Aβ are capable of impacting on behavioural symptoms of AD, at least in rodent models of the disease. *In vivo* studies allow the phenotypic effects of flavonoid molecules to be elucidated; however they have not provided mechanistic insight into the actions of flavonoids on specific cell types or cellular pathways.

*In vitro* studies, such as those conducted in this thesis, have allowed the effects of flavonoids on neuronal cells to be studied and have shown that select flavonoids are able to reduce amyloidogenic APP processing (Chapter 4) and (Obregon et al., 2006, Wang et al., 2006, Rezai-Zadeh et al., 2009, Ho et al., 2013)) (Figure 7.1(A)). It appears that flavonoids have multiple actions on APP processing depending on the concentrations involved. In this study the flavanols (-) epicatechin and epigallocatechin indirectly inhibited BACE1 activity at low concentrations (Figure 7.1(B)). In independent studies, another catechin, EGCG, and a catechin-rich Cabernet Sauvignon extract have been shown to promote α-secretase-mediated APP processing at high concentrations, increasing levels of sAPPα (Rezai-Zadeh, 2005, Wang et al., 2006) (Figure 7.1(C)).

Concentration dependence is also crucial for actions of flavonoids at other cellular targets. Low concentrations of (-) epicatechin activate ERK activity, with maximal effect at 100 nM (Chapter 6) and (Schroeter et al., 2007)) (Figure 7.1(D)), however it appears that only mid-micromolar concentrations may potentiate NMDA-mediated ERK dependent transcriptional activity (Chapter 6) (Figure 7.1(E)). This suggests that at physiological concentrations achievable in the brain, (-) epicatechin modulation of ERK activity is likely at a cytoplasmic target rather than through translocation to the nucleus and gene transcription. Fisetin was also suggested to be a regulator of ERK-dependent transcriptional activity at micromolar concentrations however, in contrast to (-) epicatechin, showing a trend towards reducing ERK-dependent transcriptional activity (Chapter 6) (Figure 7.2(F)). Although this result is in contrast

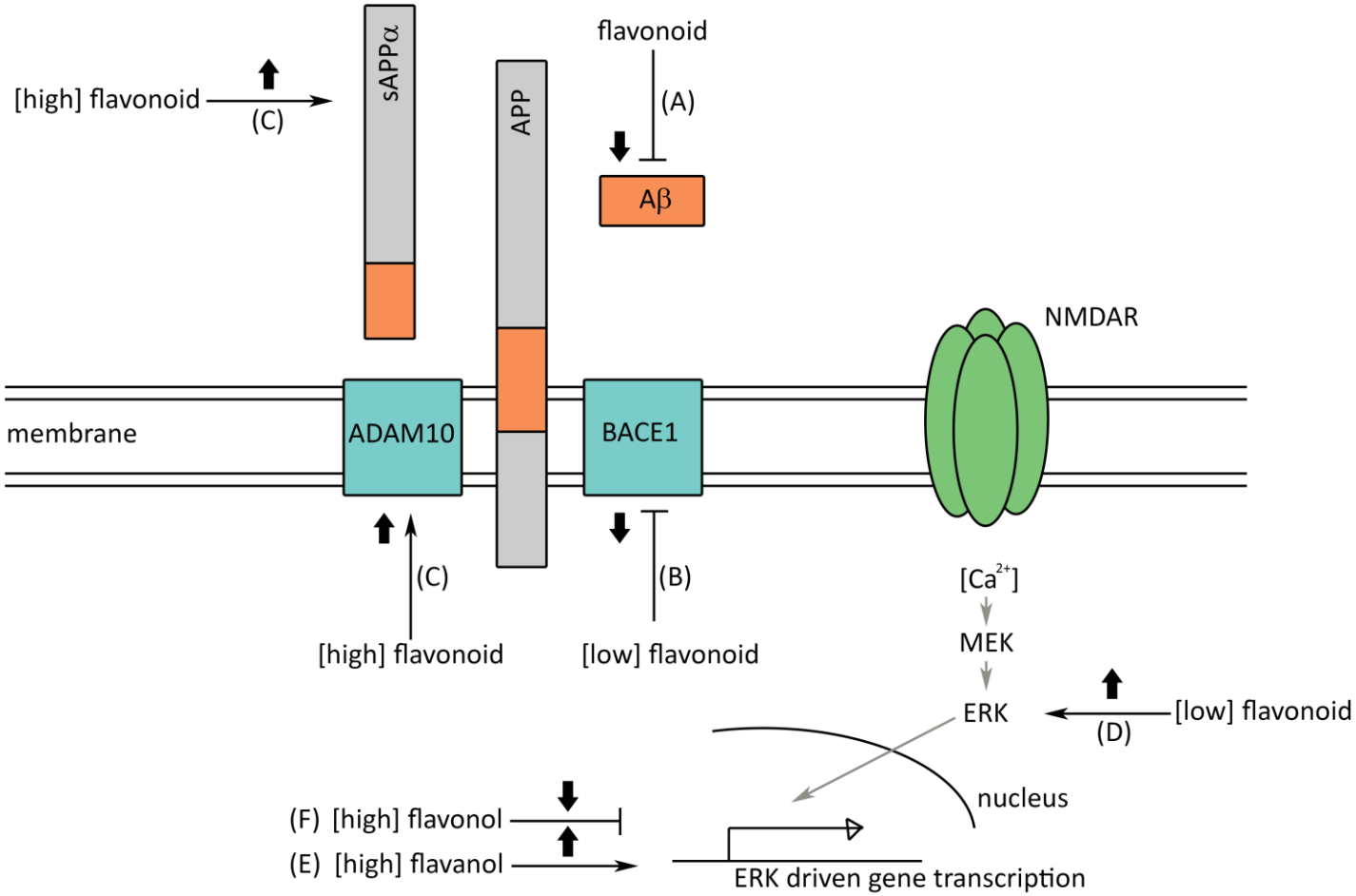

[high] flavonoid
(C)
sAPPα
APP
flavonoid
(A)
Aβ
NMDAR
membrane
ADAM10
BACE1
(C)
[high] flavonoid
(B)
[low] flavonoid
[Ca2+]
MEK
ERK
(D)
[low] flavonoid
nucleus
(F) [high] flavonol
(E) [high] flavanol
ERK driven gene transcription

Figure 7.1: **Model of flavonoid modulation of APP processing and ERK activity in primary neurons**

The cellular effects of flavonoids are strongly dependent on concentration. The multimodal activities of flavonoids lead to inhibition of Aβ production (A) via a number of different mechanisms. Low concentrations of flavonoids modulate BACE1 activity (B) and activity of ERK (D). High concentrations of flavonoids modulate ADAM10 expression and sAPPα levels (C) as well as NMDA mediated ERK driven gene transcription (D and E). Thick black arrows indicate flavonoid mediated effect on activity. [ ] - concentration

to previous work identifying fisetin as an activator of the ERK pathway (Maher et al., 2006), it is supported by data from another flavonol, quercetin, identified as a potent inhibitor of another kinase - PI3K, through binding to its ATP binding domain (Vlahos et al., 1994). These contrasting results could be as a result of concentration-dependent activities. It is tempting to propose that these potential differential activities at ERK are linked to the biphasic activity of fisetin seen at APP processing (Chapter 4) as inhibition of ERK signalling can lead to increased amyloidogenic processing (Rohe et al., 2009, Gil-Bea et al., 2012). This further underlines how imperative it is consider concentration when comparing flavonoid-mediated effects between studies.

It is unclear whether the BACE1 inhibition and the suggested potentiation of ERK activity by (-) epicatechin are part of the same mechanism, with activation of ERK upstream of BACE1 inhibition. Epigallocatechin was also an indirect inhibitor of BACE1 activity, however it did not affect ERK activity at any concentration, suggesting the two mechanisms are independent. However, neuronal activity-dependent ERK activation has been shown to down-regulate amyloidogenic processing (Hoey et al., 2009, Verges et al., 2011). This could be taken as evidence for a potential link between the two activities. However, these studies showed ERK activation led to increased levels of sAPPα, which was not altered by (-) epicatechin in this study (Chapter 5). (-) Epicatechin has been shown to exert multiple cellular effects, which may act synergistically to decrease amyloidogenic processing in primary neurons.

Although (-) epicatechin was the focus of this thesis, the four flavonoids identified in the original screen are also potentially interesting molecules as inhibitors of amyloidogenic APP processing. (-) Epicatechin was the focus of this study due to its high potency, known blood-brain barrier permeability and good oral bioavailability properties. Fisetin has poor bioavailability due to poor water solubility, forming a significant barrier to development as an effective *in vivo* inhibitor. However, attempts to design liposomal formulations of fisetin have had success, increasing bioavailability 47-fold whilst maintaining efficacy (Mignet et al., 2012, Seguin et al., 2013), suggesting packaging of flavonoids is a viable approach to overcome their

poor solubility properties. Bioavailability of pelargonidin in humans has not been fully assessed, although presence of the pelargonidin aglycone in plasma following ingestion of strawberries has been demonstrated (Azzini et al., 2010). No human bioavailability data is available for sinensetin, however an oral delivery study in rat suggests it is very poorly absorbed (Loon et al., 2005). Sinensetin remains an interesting molecule however as it was the only flavonoid to inhibit amyloidogenic processing at both concentrations tested (chapter 4). Sinensetin was the only flavonoid tested with methoxy- substitutions on its central structure. Flavonoids with methoxy- substitutions have been shown to have increased metabolic stability compared to hydroxylated compounds. Metabolism of active flavonoids to inactive metabolites in the small intestine and liver significantly affects activity of many bioavailable flavonoids (Spencer et al., 2001, Abd El Mohsen et al., 2002, El Mohsen et al., 2006), therefore the relative stability of sinensetin could make it a better drug candidate. Epigallocatechin, despite its extremely similar structure to (-) epicatechin, has greatly reduced bioavailability in comparison, although levels are still detectable in human plasma following oral ingestion (Renouf et al., 2013). The biphasic activity at APP processing of epigallocatechin, as well as fisetin, also raises an important consideration for future flavonoid activity studies, as flavonoid concentration may need to be strictly controlled to avoid off-target negative side effects (Chapter 4).

Treatments currently available for AD patients are symptomatics and therefore do not alter the underlying pathophysiology of the disease. AChEIs were the first class of agents to be approved for the treatment of AD, acting to increase the availability of ACh at cholinergic synapses. Currently three are widely licenced for treatment of mild to moderate AD: donepezil, rivastigmine and galantamine (Wilcock et al., 2000, Seltzer et al., 2004, Cummings et al., 2007). These small molecule drugs aim to maintain neuronal activity in affected brain areas, which has been shown to reduce the rate of cognitive decline compared to placebo (Rogers and Friedhoff, 1996, Rosler et al., 1999, Raskind et al., 2000). Another small molecule that has been FDA approved is memantine, which is a non-competitive antagonist of NMDAR and acts to attenuate NMDAR function, aiming to slow neuronal loss in medium to late stage

dementia (Winblad and Poritis, 1999). Although popular, like all drugs currently available to AD sufferers, memantine is not a disease-modifying drug and can only alleviate symptoms (Herrmann et al., 2011).

Currently there are 60 therapeutics in active clinical trials for the treatment of AD (Alzheimer's Research Forum accessed 27/02/2014). Due to the central role of Aβ in AD pathogenesis, it has become the main drug target for the development of disease modifying treatments. There are two principal therapeutic strategies: small molecule inhibitors of Aβ production or Aβ vaccinations to promote clearance of aggregated Aβ from the brain (Schenk et al., 2012).

Inhibition of Aβ production has focussed on BACE1 and the γ-secretase complex as the obvious drug targets. The largest hurdle to overcome in the development of γ-secretase inhibitors are the side effects due to other γ-secretase substrates, most notably notch. Semagacestat, the most advanced non-selective inhibitor, was halted during Phase III human testing due to a high occurrence of adverse events reported associated with notch inhibition and no observed improvement in cognitive (Doody et al., 2013). This led to the development of two new classes of γ-secretase inhibitor: γ-secretase modulators (GSM) based on NSAIDs and notch sparing/APP selective γ-secretase inhibitors (GSI). Both of these however have also suffered from disappointing results. GSMs such as R-flurbuprofen have been discontinued to due lack of efficacy and notch sparing GSIs such as BMS-299897 have also shown disappointing efficacy. These have also caused unacceptable side effects such as ELND006 which showed dose-limiting liver toxicity (Schenk et al., 2012).

Targeting BACE has also proved difficult due to its extended substrate binding groove and multiple substrates. Selectivity over other aspartyl proteases has also been an issue. Development of CNS permeable, selective molecules has been slow, with few compounds making it to clinical development; only two trials are currently active. MK-8931, the furthest through development is a BACE1 and BACE2 inhibitor currently in Phase III trials following good tolerability and dose-dependent reductions of Aβ results from Phase II.

The second area of drug development that has received much attention is Aβ immunotherapy, following a seminal paper in PDAPP transgenic mice demonstrating IP injection of Aβ once a month for a year led to reduced plaque burden, reduced neuritic dystrophy and associated inflammatory changes (Schenk et al., 1999). The first Aβ immunisation trial developed by Elan and Wyeth, AN1792, reduced Aβ levels in plasma and CSF but failed in Phase II as 6% of patients developed ameningoencephalitis and leukoencephalopathy (Orgogozo et al., 2003). This was an active immunisation using full length $A\beta_{1-42}$ and caused the field to shift towards passive immunisation with humanised antibodies rather than the full length Aβ peptide. These have proven to be safer, with less adverse events reported (Lemere and Masliah, 2010). Solanezumab by Eli Lilly reached Phase III trials for mild to moderate AD, however failed due to no improvement in cognition or functional ability at the end of the study (Doody et al., 2014). Further analysis of the data, however showed a small benefit in a subset of mild AD patients and Eli Lilly have announced a new Phase III trial in patients with mild AD (www.clinicaltrials.gov). The positive effect of Solanezumab in mild AD patients has prompted Genentech to conduct the first trial of passive immunotherapy in a prevention paradigm: Crenezumab will be given to preclinical carriers of the PSEN E280A autosomal dominant mutation in a Colombian cohort of patients (www.clinicaltrials.gov). This study is still in its recruitment stage, however will be crucial in determining the potential of APP-targeting drugs in the treatment of AD and a stern test of the amyloid cascade hypothesis of AD. Immunisation has potential as a viable therapeutic approach for those at high risk of FAD however, due to the current lack of accurate biomarkers and good diagnostic tools for AD, immunisation is a treatment that most AD patients would currently miss out on as LOAD is often not diagnosed until pathology and symptoms are well established.

The current failure rate of AD drugs has driven research interests towards alternative AD therapy approaches. An emerging consensus that treatment of AD is required much earlier in the disease process emphasises the needed for safer treatments. This thesis has provided further evidence that select flavonoids reduce amyloidogenic processing in primary neuronal cells through multimodal activities.

(-) Epicatechin and its metabolites show good bioavailability both in rodents and in humans and reach quantifiable concentrations in the brain (Richelle et al., 1999, Abd El Mohsen et al., 2002, van Praag et al., 2007, Wang et al., 2012, Renouf et al., 2013). As a dietary molecule it is well tolerated with no toxic effects reported. Its inhibitory action at APP is independent of notch, a significant hurdle in the AD therapy field (Chapter 5). (-) Epicatechin is a potent inhibitor of amyloidogenic APP processing at physiologically relevant, nanomolar concentrations, causing a 20% reduction in processing in WT primary neurons (Chapter 4). In collaborative work this *in vitro* activity was confirmed in preliminary *in vivo* work in the TASTPM transgenic mouse model of AD, whereby oral (-) epicatechin treatment led to decreased amyloid pathology (Cox et al., under review). Oral delivery of (-) epicatechin in two independent studies has led to cognitive improvements, one in a mouse model of AD (van Praag et al., 2007, Wang et al., 2012), indicating the (-) epicatechin-mediated effects are having a positive impact on cognition and function, at least in rodents. It remains to be seen whether the (-) epicatechin mediated improvements observed in rodent models can translate to cognitive benefits in man and furthermore whether these benefits would be sufficient for (-) epicatechin to be an effective prophylactic for the prevention of AD.

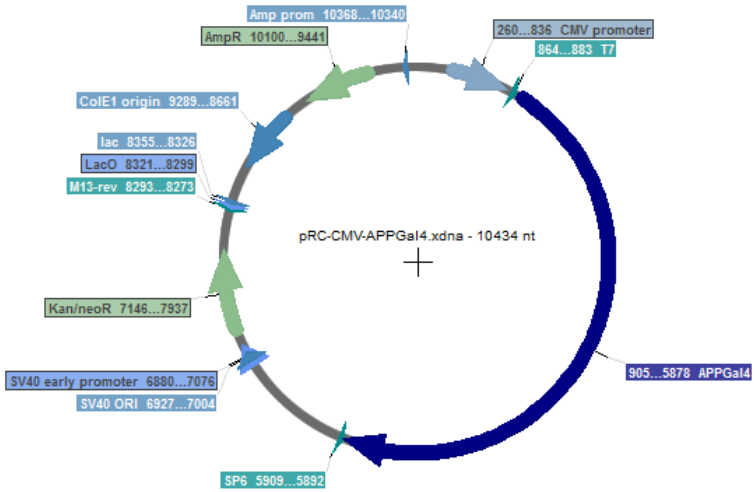
These questions are extremely important given the scarcity of effective therapies and when no disease modifying drugs are available. Shifts in focus to earlier intervention means conventional drug therapy will have to be safer, thus more difficult to develop and further from entering the clinic. Development of effective preventative treatments would provide the growing economic and social burden of potential AD patients with a line of proven defence against this disease. For this reason, clinical trials of flavonoids must be tightly controlled and strictly regulated so that previous inconsistencies in flavonoid studies can be avoided and a clear message can be communicated.

# 7.2. Conclusion

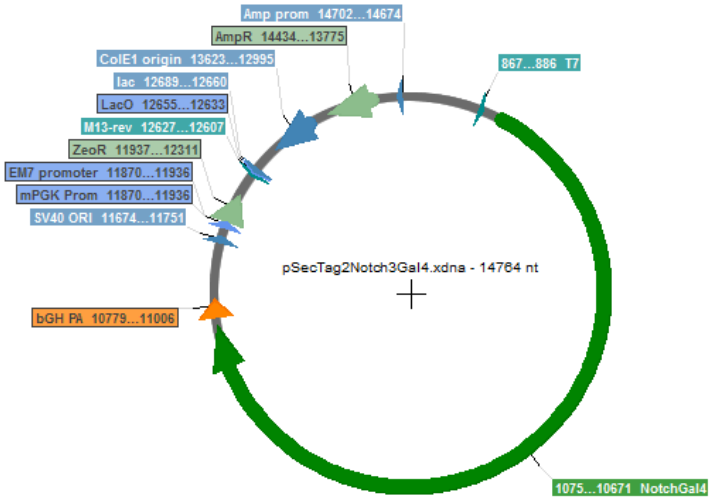
This study confirmed that select flavonoids are able to reduce amyloidogenic processing in primary cortical neurons. Investigation into the mechanism of inhibition by (-) epicatechin suggests that the mechanism is likely through indirect BACE1 inhibition. The study of ERK-regulation activities by the lead flavonoids suggested (-) epicatechin can also activate ERK activity and that fisetin may inhibit ERK-dependent transcriptional activity. This study supports the hypothesis that flavonoids are modulators of APP processing, achieved through multi-modal activities that are spread across the families.

# Appendices

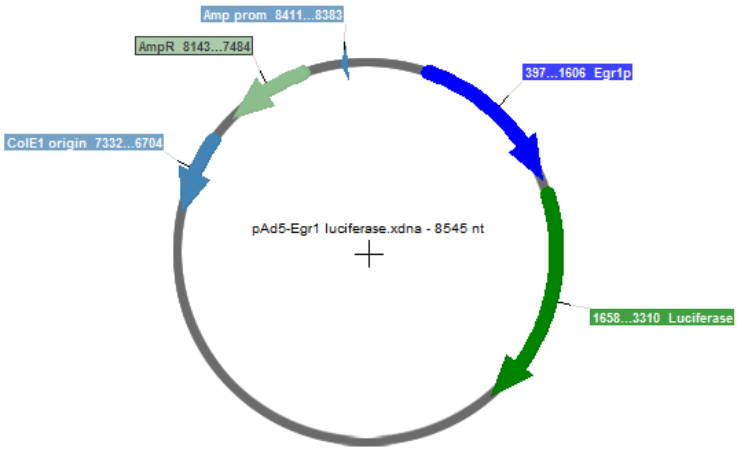
## Appendix 1: **Plasmid map of APP-Gal4**




## Appendix 2: **Plasmid map of notch-Gal4**



# Appendix 3: **Plasmid map of Egr1- luciferase**



# References


Abd El Mohsen MM, Kuhnle G, Rechner AR, Schroeter H, Rose S, Jenner P, Rice-Evans CA (2002) Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. Free Radic Biol Med 33:1693-1702.

Acx H, Chavez-Gutierrez L, Serneels L, Lismont S, Benurwar M, Elad N, De Strooper B (2013) Signature Abeta Profiles Are Produced by Different gamma-Secretase Complexes. J Biol Chem.

Adams JP, Roberson ED, English JD, Selcher JC, Sweatt JD (2000) MAPK regulation of gene expression in the central nervous system. Acta neurobiologiae experimentalis 60:377-394.

Adlerz L, Holback S, Multhaup G, Iverfeldt K (2007) IGF-1-induced processing of the amyloid precursor protein family is mediated by different signaling pathways. J Biol Chem 282:10203-10209.

Aletrino MA, Vogels OJ, Van Domburg PH, Ten Donkelaar HJ (1992) Cell loss in the nucleus raphes dorsalis in Alzheimer's disease. Neurobiol Aging 13:461-468.

Alzheimer A, Stelzman R, Schnitzlein H, Murtagh F (1995) An english translation of Alzheimer's 1907 paper "Uber eine eigenartige Erkankung der Hirnrinde". Clin Anat 8:429-431.

Amour A, Knight CG, Webster A, Slocombe PM, Stephens PE, Knauper V, Docherty AJ, Murphy G (2000) The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. FEBS Lett 473:275-279.

Ancolio K, Dumanchin C, Barelli H, Warter JM, Brice A, Campion D, Frebourg T, Checler F (1999) Unusual phenotypic alteration of beta amyloid precursor protein (betaAPP) maturation by a new Val-715 --> Met betaAPP-770 mutation responsible for probable early-onset Alzheimer's disease. Proc Natl Acad Sci U S A 96:4119-4124.

Apostolova LG, Lu P, Rogers S, Dutton RA, Hayashi KM, Toga AW, Cummings JL, Thompson PM (2008) 3D mapping of language networks in clinical and pre-clinical Alzheimer's disease. Brain and language 104:33-41.

Apostolova LG, Lu PH, Rogers S, Dutton RA, Hayashi KM, Toga AW, Cummings JL, Thompson PM (2006) 3D mapping of mini-mental state examination performance in clinical and preclinical Alzheimer disease. Alzheimer Dis Assoc Disord 20:224-231.

Apostolova LG, Steiner CA, Akopyan GG, Dutton RA, Hayashi KM, Toga AW, Cummings JL, Thompson PM (2007) Three-dimensional gray matter atrophy mapping in mild cognitive impairment and mild Alzheimer disease. Arch Neurol 64:1489-1495.

Araki Y, Tomita S, Yamaguchi H, Miyagi N, Sumioka A, Kirino Y, Suzuki T (2003) Novel cadherin-related membrane proteins, Alcadeins, enhance the X11-like protein-mediated stabilization of amyloid beta-protein precursor metabolism. J Biol Chem 278:49448-49458.

Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284:770-776.

Azzini E, Vitaglione P, Intorre F, Napolitano A, Durazzo A, Foddai MS, Fumagalli A, Catasta G, Rossi L, Venneria E, Raguzzini A, Palomba L, Fogliano V, Maiani G (2010) Bioavailability of strawberry antioxidants in human subjects. The British journal of nutrition 104:1165-1173.

Back S, Haas P, Tschape JA, Gruebl T, Kirsch J, Muller U, Beyreuther K, Kins S (2007) beta-amyloid precursor protein can be transported independent of any sorting signal to the axonal and dendritic compartment. J Neurosci Res 85:2580-2590.

Bahia PK, Rattray M, Williams RJ (2008) The dietary flavonoid (-)epicatechin stimulates phosphatidylinositol 3-kinase dependent antioxidant response element activity and upregulates glutathione in cortical astrocytes. Journal of Neurochemistry.

Bai G, Chivatakarn O, Bonanomi D, Lettieri K, Franco L, Xia C, Stein E, Ma L, Lewcock JW, Pfaff SL (2011) Presenilin-dependent receptor processing is required for axon guidance. Cell 144:106-118.

Bandyopadhyay S, Goldstein LE, Lahiri DK, Rogers JT (2007) Role of the APP non-amyloidogenic signaling pathway and targeting alpha-secretase as an alternative drug target for treatment of Alzheimer's disease. Current medicinal chemistry 14:2848-2864.

Barberger-Gateau P, Raffaitin C, Letenneur L, Berr C, Tzourio C, Dartigues JF, Alperovitch A (2007) Dietary patterns and risk of dementia: the Three-City cohort study. Neurology 69:1921-1930.

Bartus RT, Dean RL, 3rd, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408-414.

Beckett C, Nalivaeva NN, Belyaev ND, Turner AJ (2011) Nuclear signalling by membrane protein intracellular domains: The AICD enigma. Cell Signal.

Beher D, Hesse L, Masters CL, Multhaup G (1996) Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I. J Biol Chem 271:1613-1620.

Bell RD, Deane R, Chow N, Long X, Sagare A, Singh I, Streb JW, Guo H, Rubio A, Van Nostrand W, Miano JM, Zlokovic BV (2009) SRF and myocardin regulate LRP-mediated amyloid-beta clearance in brain vascular cells. Nat Cell Biol 11:143-153.

Bell RD, Sagare AP, Friedman AE, Bedi GS, Holtzman DM, Deane R, Zlokovic BV (2007) Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. J Cereb Blood Flow Metab 27:909-918.

Belyaev ND, Kellett KAB, Beckett C, Makova NZ, Revett TJ, Nalivaeva NN, Hooper NM, Turner AJ (2010) The Transcriptionally Active Amyloid Precursor Protein (APP) Intracellular Domain Is Preferentially Produced from the 695 Isoform of APP in a  -Secretase-dependent Pathway. Journal of Biological Chemistry 285:41443-41454.

Ben Mahdi MH, Andrieu V, Pasquier C (2000) Focal adhesion kinase regulation by oxidative stress in different cell types. IUBMB Life 50:291-299.

Bennett BD, Denis P, Haniu M, Teplow DB, Kahn S, Louis JC, Citron M, Vassar R (2000) A furin-like convertase mediates propeptide cleavage of BACE, the Alzheimer's beta -secretase. J Biol Chem 275:37712-37717.

Bentahir M, Nyabi O, Verhamme J, Tolia A, Horre K, Wiltfang J, Esselmann H, De Strooper B (2006) Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. J Neurochem 96:732-742.

Bergmans BA, De Strooper B (2010) gamma-secretases: from cell biology to therapeutic strategies. Lancet Neurol 9:215-226.

Bero AW, Yan P, Roh JH, Cirrito JR, Stewart FR, Raichle ME, Lee JM, Holtzman DM (2011) Neuronal activity regulates the regional vulnerability to amyloid-beta deposition. Nat Neurosci 14:750-756.

Bertram L, Lill CM, Tanzi RE (2010) The genetics of Alzheimer disease: back to the future. Neuron 68:270-281.

Bertrand E, Brouillet E, Caille I, Bouillot C, Cole GM, Prochiantz A, Allinquant B (2001) A short cytoplasmic domain of the amyloid precursor protein induces apoptosis in vitro and in vivo. Mol Cell Neurosci 18:503-511.

Beyreuther K, Multhaup G, Monning U, Sandbrink R, Beher D, Hesse L, Small DH, Masters CL (1996) Regulation of APP expression, biogenesis and metabolism by extracellular matrix and cytokines. Ann N Y Acad Sci 777:74-76.

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385:729-733.

Bloodgood BL, Sabatini BL (2007) Ca(2+) signaling in dendritic spines. Curr Opin Neurobiol 17:345-351.

Bordji K, Becerril-Ortega J, Nicole O, Buisson A (2010) Activation of Extrasynaptic, But Not Synaptic, NMDA Receptors Modifies Amyloid Precursor Protein

Expression Pattern and Increases Amyloid- Production. Journal of Neuroscience 30:15927-15942.

Bors W, Heller W, Michel C, Saran M (1990) Radical Chemistry of Flavonoid Antioxidants. Antioxidants in Therapy and Preventive Medicine 264:165-170.

Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239-259.

Brinkmalm G, Brinkmalm A, Bourgeois P, Persson R, Hansson O, Portelius E, Mercken M, Andreasson U, Parent S, Lipari F, Ohrfelt A, Bjerke M, Minthon L, Zetterberg H, Blennow K, Nutu M (2013) Soluble amyloid precursor protein alpha and beta in CSF in Alzheimer's disease. Brain Res 1513:117-126.

Brown MS, Ye J, Rawson RB, Goldstein JL (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell 100:391-398.

Bugiani O, Giaccone G, Rossi G, Mangieri M, Capobianco R, Morbin M, Mazzoleni G, Cupidi C, Marcon G, Giovagnoli A, Bizzi A, Di Fede G, Puoti G, Carella F, Salmaggi A, Romorini A, Patruno GM, Magoni M, Padovani A, Tagliavini F (2010) Hereditary cerebral hemorrhage with amyloidosis associated with the E693K mutation of APP. Arch Neurol 67:987-995.

Burg VK, Grimm HS, Rothhaar TL, Grosgen S, Hundsdorfer B, Haupenthal VJ, Zimmer VC, Mett J, Weingartner O, Laufs U, Broersen LM, Tanila H, Vanmierlo T, Lutjohann D, Hartmann T, Grimm MO (2013) Plant sterols the better cholesterol in Alzheimer's disease? A mechanistical study. J Neurosci 33:16072-16087.

Bushlin I, Petralia RS, Wu F, Harel A, Mughal MR, Mattson MP, Yao PJ (2008) Clathrin assembly protein AP180 and CALM differentially control axogenesis and dendrite outgrowth in embryonic hippocampal neurons. J Neurosci 28:10257-10271.

Buxbaum JD, Liu KN, Luo Y, Slack JL, Stocking KL, Peschon JJ, Johnson RS, Castner BJ, Cerretti DP, Black RA (1998) Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. J Biol Chem 273:27765-27767.

Caccamo A, Fisher A, LaFerla FM (2009) M1 agonists as a potential disease-modifying therapy for Alzheimer's disease. Current Alzheimer research 6:112-117.

Caceres J, Brandan E (1997) Interaction between Alzheimer's disease beta A4 precursor protein (APP) and the extracellular matrix: evidence for the participation of heparan sulfate proteoglycans. J Cell Biochem 65:145-158.

Cai J, Jiang WG, Grant MB, Boulton M (2006) Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. J Biol Chem 281:3604-3613.

Cai XD, Golde TE, Younkin SG (1993) Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. Science 259:514-516.

Caille I, Allinquant B, Dupont E, Bouillot C, Langer A, Muller U, Prochiantz A (2004) Soluble form of amyloid precursor protein regulates proliferation of progenitors in the adult subventricular zone. Development 131:2173-2181.

Campbell DS, Holt CE (2003) Apoptotic pathway and MAPKs differentially regulate chemotropic responses of retinal growth cones. Neuron 37:939-952.

Campion D, Dumanchin C, Hannequin D, Dubois B, Belliard S, Puel M, Thomas-Anterion C, Michon A, Martin C, Charbonnier F, Raux G, Camuzat A, Penet C, Mesnage V, Martinez M, Clerget-Darpoux F, Brice A, Frebourg T (1999) Early-onset autosomal dominant Alzheimer disease: prevalence, genetic heterogeneity, and mutation spectrum. Am J Hum Genet 65:664-670.

Cao X, Sudhof TC (2001) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 293:115-120.

Cao X, Sudhof TC (2004) Dissection of amyloid-beta precursor protein-dependent transcriptional transactivation. J Biol Chem 279:24601-24611.

Caporaso GL, Takei K, Gandy SE, Matteoli M, Mundigl O, Greengard P, De Camilli P (1994) Morphologic and biochemical analysis of the intracellular trafficking of the Alzheimer beta/A4 amyloid precursor protein. J Neurosci 14:3122-3138.

Casella L, Gullotti M (1993) Dioxygen activation by biomimetic dinuclear complexes. In Bioinorganic Chemistry of Copper,. New York: Chapmann & Hall.

Caunt CJ, Rivers CA, Conway-Campbell BL, Norman MR, McArdle CA (2008) Epidermal growth factor receptor and protein kinase C signaling to ERK2: spatiotemporal regulation of ERK2 by dual specificity phosphatases. J Biol Chem 283:6241-6252.

Chami L, Checler F (2012) BACE1 is at the crossroad of a toxic vicious cycle involving cellular stress and beta-amyloid production in Alzheimer's disease. Mol Neurodegener 7:52.

Chang KA, Kim HS, Ha TY, Ha JW, Shin KY, Jeong YH, Lee JP, Park CH, Kim S, Baik TK, Suh YH (2006) Phosphorylation of amyloid precursor protein (APP) at Thr668 regulates the nuclear translocation of the APP intracellular domain and induces neurodegeneration. Molecular and cellular biology 26:4327-4338.

Chen CP, Eastwood SL, Hope T, McDonald B, Francis PT, Esiri MM (2000) Immunocytochemical study of the dorsal and median raphe nuclei in

patients with Alzheimer's disease prospectively assessed for behavioural changes. Neuropathology and applied neurobiology 26:347-355.

Chen F, Hasegawa H, Schmitt-Ulms G, Kawarai T, Bohm C, Katayama T, Gu Y, Sanjo N, Glista M, Rogaeva E, Wakutani Y, Pardossi-Piquard R, Ruan X, Tandon A, Checler F, Marambaud P, Hansen K, Westaway D, St George-Hyslop P, Fraser P (2006) TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. Nature 440:1208-1212.

Choudhry F, Howlett DR, Richardson JC, Francis PT, Williams RJ (2012) Pro-oxidant diet enhances beta/gamma secretase-mediated APP processing in APP/PS1 transgenic mice. Neurobiol Aging 33:960-968.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672-674.

Citron M, Vigo-Pelfrey C, Teplow DB, Miller C, Schenk D, Johnston J, Winblad B, Venizelos N, Lannfelt L, Selkoe DJ (1994) Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. Proc Natl Acad Sci U S A 91:11993-11997.

Clark RF, Hutton M, Talbot C, Wragg M, Lendon C, Busfield F, Han SW, Perez-Tur J, Adams M, Fuldner R, Roberts G, Karran E, Hardy J, Goate A (1996) The role of presenilin 1 in the genetics of Alzheimer's disease. Cold Spring Harb Symp Quant Biol 61:551-558.

Colombo A, Wang H, Kuhn PH, Page R, Kremmer E, Dempsey PJ, Crawford HC, Lichtenthaler SF (2012) Constitutive alpha- and beta-secretase cleavages of the amyloid precursor protein are partially coupled in neurons, but not in frequently used cell lines. Neurobiol Dis 49C:137-147.

Comas-Herrera A, Wittenberg R, Pickard L, Knapp M (2007) Cognitive impairment in older people: future demand for long-term care services and the associated costs. Int J Geriatr Psychiatry 22:1037-1045.

Commenges D, Scotet V, Renaud S, Jacqmin-Gadda H, Barberger-Gateau P, Dartigues JF (2000) Intake of flavonoids and risk of dementia. Eur J Epidemiol 16:357-363.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261:921-923.

Cruts M, Dermaut B, Rademakers R, Van den Broeck M, Stogbauer F, Van Broeckhoven C (2003) Novel APP mutation V715A associated with presenile Alzheimer's disease in a German family. J Neurol 250:1374-1375.

Cruts M, Theuns J, Van Broeckhoven C (2012) Locus-specific mutation databases for neurodegenerative brain diseases. Hum Mutat 33:1340-1344.

Cruts M, Van Broeckhoven C (1998) Molecular genetics of Alzheimer's disease. Ann Med 30:560-565.

Cummings J, Lefevre G, Small G, Appel-Dingemanse S (2007) Pharmacokinetic rationale for the rivastigmine patch. Neurology 69:S10-13.

Currais A, Prior M, Dargusch R, Armando A, Ehren J, Schubert D, Quehenberger O, Maher P (2013) Modulation of p25 and inflammatory pathways by fisetin maintains cognitive function in Alzheimer's disease transgenic mice. Aging cell.

Dai Q, Borenstein AR, Wu Y, Jackson JC, Larson EB (2006) Fruit and vegetable juices and Alzheimer's disease: the Kame Project. Am J Med 119:751-759.

Dajas F, Rivera F, Blasina F, Arredondo F, Echeverry C, Lafon L, Morquio A, Heinzen H (2003) Cell culture protection and in vivo neuroprotective capacity of flavonoids. Neurotoxicity research 5:425-432.

Das U, Scott DA, Ganguly A, Koo EH, Tang Y, Roy S (2013) Activity-induced convergence of APP and BACE-1 in acidic microdomains via an endocytosis-dependent pathway. Neuron 79:447-460.

Davis S, Laroche S (2006) Mitogen-activated protein kinase/extracellular regulated kinase signalling and memory stabilization: a review. Genes, brain, and behavior 5 Suppl 2:61-72.

De Jonghe C, Zehr C, Yager D, Prada CM, Younkin S, Hendriks L, Van Broeckhoven C, Eckman CB (1998) Flemish and Dutch mutations in amyloid beta precursor protein have different effects on amyloid beta secretion. Neurobiol Dis 5:281-286.

De Strooper B (2010) Proteases and proteolysis in Alzheimer disease: a multifactorial view on the disease process. Physiol Rev 90:465-494.

De Strooper B, Annaert W (2000) Proteolytic processing and cell biological functions of the amyloid precursor protein. J Cell Sci 113 ( Pt 11):1857-1870.

De Strooper B, Iwatsubo T, Wolfe MS (2012) Presenilins and gamma-secretase: structure, function, and role in Alzheimer Disease. Cold Spring Harb Perspect Med 2:a006304.

De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature 391:387-390.

De Strooper B, Vassar R, Golde T (2010) The secretases: enzymes with therapeutic potential in Alzheimer disease. Nature Reviews Neurology 6:99-107.

Dehmelt L, Halpain S (2005) The MAP2/Tau family of microtubule-associated proteins. Genome biology 6:204.

Demars MP, Bartholomew A, Strakova Z, Lazarov O (2011) Soluble amyloid precursor protein: a novel proliferation factor of adult progenitor cells of ectodermal and mesodermal origin. Stem cell research & therapy 2:36.

DeMattos RB, Cirrito JR, Parsadanian M, May PC, O'Dell MA, Taylor JW, Harmony JA, Aronow BJ, Bales KR, Paul SM, Holtzman DM (2004) ApoE and clusterin cooperatively suppress Abeta levels and deposition: evidence that ApoE regulates extracellular Abeta metabolism in vivo. Neuron 41:193-202.

Devore EE, Kang JH, Breteler MM, Grodstein F (2012) Dietary intakes of berries and flavonoids in relation to cognitive decline. Ann Neurol 72:135-143.

Dolcini V, Dunys J, Sevalle J, Chen F, Guillot-Sestier MV, St George-Hyslop P, Fraser PE, Checler F (2008) TMP21 regulates Abeta production but does not affect caspase-3, p53, and neprilysin. Biochem Biophys Res Commun 371:69-74.

Donoviel DB, Hadjantonakis AK, Ikeda M, Zheng H, Hyslop PS, Bernstein A (1999) Mice lacking both presenilin genes exhibit early embryonic patterning defects. Genes & development 13:2801-2810.

Doody RS, Raman R, Farlow M, Iwatsubo T, Vellas B, Joffe S, Kieburtz K, He F, Sun X, Thomas RG, Aisen PS, Siemers E, Sethuraman G, Mohs R (2013) A phase 3 trial of semagacestat for treatment of Alzheimer's disease. The New England journal of medicine 369:341-350.

Doody RS, Thomas RG, Farlow M, Iwatsubo T, Vellas B, Joffe S, Kieburtz K, Raman R, Sun X, Aisen PS, Siemers E, Liu-Seifert H, Mohs R (2014) Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. The New England journal of medicine 370:311-321.

Dulin F, Leveille F, Ortega JB, Mornon JP, Buisson A, Callebaut I, Colloc'h N (2008) P3 peptide, a truncated form of A beta devoid of synaptotoxic effect, does not assemble into soluble oligomers. FEBS Lett 582:1865-1870.

Eckel-Mahan KL, Phan T, Han S, Wang H, Chan GC, Scheiner ZS, Storm DR (2008) Circadian oscillation of hippocampal MAPK activity and cAmp: implications for memory persistence. Nat Neurosci 11:1074-1082.

Eckman CB, Mehta ND, Crook R, Perez-tur J, Prihar G, Pfeiffer E, Graff-Radford N, Hinder P, Yager D, Zenk B, Refolo LM, Prada CM, Younkin SG, Hutton M, Hardy J (1997) A new pathogenic mutation in the APP gene (I716V) increases the relative proportion of A beta 42(43). Hum Mol Genet 6:2087-2089.

Eckman EA, Reed DK, Eckman CB (2001) Degradation of the Alzheimer's amyloid beta peptide by endothelin-converting enzyme. J Biol Chem 276:24540-24548.

Ehrnhoefer DE, Bieschke J, Boeddrich A, Herbst M, Masino L, Lurz R, Engemann S, Pastore A, Wanker EE (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. Nature structural & molecular biology 15:558-566.

El Mohsen MA, Marks J, Kuhnle G, Moore K, Debnam E, Kaila Srai S, Rice-Evans C, Spencer JP (2006) Absorption, tissue distribution and excretion of pelargonidin and its metabolites following oral administration to rats. The British journal of nutrition 95:51-58.

Endres K, Fahrenholz F (2010) Upregulation of the alpha-secretase ADAM10--risk or reason for hope? FEBS J 277:1585-1596.

Esch FS, Keim PS, Beattie EC, Blacher RW, Culwell AR, Oltersdorf T, McClure D, Ward PJ (1990) Cleavage of amyloid beta peptide during constitutive processing of its precursor. Science 248:1122-1124.

Ewers M, Frisoni GB, Teipel SJ, Grinberg LT, Amaro E, Jr., Heinsen H, Thompson PM, Hampel H (2011) Staging Alzheimer's disease progression with multimodality neuroimaging. Prog Neurobiol 95:535-546.

Feart C, Samieri C, Rondeau V, Amieva H, Portet F, Dartigues JF, Scarmeas N, Barberger-Gateau P (2009) Adherence to a Mediterranean diet, cognitive decline, and risk of dementia. JAMA 302:638-648.

Fernandez JW, Rezai-Zadeh K, Obregon D, Tan J (2010) EGCG functions through estrogen receptor-mediated activation of ADAM10 in the promotion of non-amyloidogenic processing of APP. FEBS Letters 584:4259-4267.

Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y, Jorm A, Mathers C, Menezes PR, Rimmer E, Scazufca M (2005) Global prevalence of dementia: a Delphi consensus study. Lancet 366:2112-2117.

Ferriola PC, Cody V, Middleton E, Jr. (1989) Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. Biochem Pharmacol 38:1617-1624.

Fiore F, Zambrano N, Minopoli G, Donini V, Duilio A, Russo T (1995) The regions of the Fe65 protein homologous to the phosphotyrosine interaction/phosphotyrosine binding domain of Shc bind the intracellular domain of the Alzheimer's amyloid precursor protein. J Biol Chem 270:30853-30856.

Fiore RS, Murphy TH, Sanghera JS, Pelech SL, Baraban JM (1993) Activation of p42 mitogen-activated protein kinase by glutamate receptor stimulation in rat primary cortical cultures. J Neurochem 61:1626-1633.

Francis PT, Ramirez MJ, Lai MK (2010) Neurochemical basis for symptomatic treatment of Alzheimer's disease. Neuropharmacology 59:221-229.

Francis R, McGrath G, Zhang J, Ruddy DA, Sym M, Apfeld J, Nicoll M, Maxwell M, Hai B, Ellis MC, Parks AL, Xu W, Li J, Gurney M, Myers RL, Himes CS, Hiebsch R, Ruble C, Nye JS, Curtis D (2002) aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev Cell 3:85-97.

Frank S, Burbach GJ, Bonin M, Walter M, Streit W, Bechmann I, Deller T (2008) TREM2 is upregulated in amyloid plaque-associated microglia in aged APP23 transgenic mice. Glia 56:1438-1447.

Frisoni GB, Ganzola R, Canu E, Rub U, Pizzini FB, Alessandrini F, Zoccatelli G, Beltramello A, Caltagirone C, Thompson PM (2008) Mapping local hippocampal changes in Alzheimer's disease and normal ageing with MRI at 3 Tesla. Brain 131:3266-3276.

Fukumori A, Fluhrer R, Steiner H, Haass C (2010) Three-amino acid spacing of presenilin endoproteolysis suggests a general stepwise cleavage of gamma-secretase-mediated intramembrane proteolysis. J Neurosci 30:7853-7862.

Fukumoto H, Cheung BS, Hyman BT, Irizarry MC (2002) Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch Neurol 59:1381-1389.

Garcia-Alloza M, Borrelli LA, Rozkalne A, Hyman BT, Bacskai BJ (2007) Curcumin labels amyloid pathology in vivo, disrupts existing plaques, and partially restores distorted neurites in an Alzheimer mouse model. J Neurochem 102:1095-1104.

Georgakopoulos A, Xu J, Xu C, Mauger G, Barthet G, Robakis NK (2011) Presenilin1/gamma-secretase promotes the EphB2-induced phosphorylation of ephrinB2 by regulating phosphoprotein associated with glycosphingolipid-enriched microdomains/Csk binding protein. FASEB J 25:3594-3604.

Gersbacher MT, Kim DY, Bhattacharyya R, Kovacs DM (2010) Identification of BACE1 cleavage sites in human voltage-gated sodium channel beta 2 subunit. Mol Neurodegener 5:61.

Gervais FG, Thornberry NA, Ruffolo SC, Nicholson DW, Roy S (1998) Caspases cleave focal adhesion kinase during apoptosis to generate a FRNK-like polypeptide. J Biol Chem 273:17102-17108.

Ghosal K, Pimplikar SW (2011) Aging and excitotoxic stress exacerbate neural circuit reorganization in amyloid precursor protein intracellular domain transgenic mice. Neurobiol Aging 32:2320 e2321-2329.

Gianni D, Zambrano N, Bimonte M, Minopoli G, Mercken L, Talamo F, Scaloni A, Russo T (2003) Platelet-derived growth factor induces the beta-gamma-secretase-mediated cleavage of Alzheimer's amyloid precursor protein through a Src-Rac-dependent pathway. J Biol Chem 278:9290-9297.

Gil-Bea FJ, Gerenu G, Aisa B, Kirazov LP, Schliebs R, Ramirez MJ (2012) Cholinergic denervation exacerbates amyloid pathology and induces hippocampal atrophy in Tg2576 mice. Neurobiol Dis 48:439-446.

Giovannini MG, Blitzer RD, Wong T, Asoma K, Tsokas P, Morrison JH, Iyengar R, Landau EM (2001) Mitogen-activated protein kinase regulates early phosphorylation and delayed expression of Ca2+/calmodulin-dependent protein kinase II in long-term potentiation. J Neurosci 21:7053-7062.

Giuffrida ML, Caraci F, Pignataro B, Cataldo S, De Bona P, Bruno V, Molinaro G, Pappalardo G, Messina A, Palmigiano A, Garozzo D, Nicoletti F, Rizzarelli E, Copani A (2009) Beta-amyloid monomers are neuroprotective. J Neurosci 29:10582-10587.

Giunta B, Hou H, Zhu Y, Salemi J, Ruscin A, Shytle RD, Tan J (2010) Fish oil enhances anti-amyloidogenic properties of green tea EGCG in Tg2576 mice. Neurosci Lett 471:134-138.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349:704-706.

Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519-526.

Goldin M, Segal M (2003) Protein kinase C and ERK involvement in dendritic spine plasticity in cultured rodent hippocampal neurons. Eur J Neurosci 17:2529-2539.

Goodger ZV, Rajendran L, Trutzel A, Kohli BM, Nitsch RM, Konietzko U (2009) Nuclear signaling by the APP intracellular domain occurs predominantly through the amyloidogenic processing pathway. Journal of Cell Science 122:3703-3714.

Grabher C, von Boehmer H, Look AT (2006) Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. Nature reviews Cancer 6:347-359.

Grabowski TJ, Cho HS, Vonsattel JP, Rebeck GW, Greenberg SM (2001) Novel amyloid precursor protein mutation in an Iowa family with dementia and severe cerebral amyloid angiopathy. Ann Neurol 49:697-705.

Gralle M, Ferreira ST (2007) Structure and functions of the human amyloid precursor protein: the whole is more than the sum of its parts. Prog Neurobiol 82:11-32.

Gu Y, Chen F, Sanjo N, Kawarai T, Hasegawa H, Duthie M, Li W, Ruan X, Luthra A, Mount HT, Tandon A, Fraser PE, St George-Hyslop P (2003) APH-1 interacts

with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin.nicastrin complexes. J Biol Chem 278:7374-7380.

Guenette S, Chang Y, Hiesberger T, Richardson JA, Eckman CB, Eckman EA, Hammer RE, Herz J (2006) Essential roles for the FE65 amyloid precursor protein-interacting proteins in brain development. EMBO J 25:420-431.

Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, Cruchaga C, Sassi C, Kauwe JS, Younkin S, Hazrati L, Collinge J, Pocock J, Lashley T, Williams J, Lambert JC, Amouyel P, Goate A, Rademakers R, Morgan K, Powell J, St George-Hyslop P, Singleton A, Hardy J (2013) TREM2 variants in Alzheimer's disease. The New England journal of medicine 368:117-127.

Guerreiro RJ, Gustafson DR, Hardy J (2012) The genetic architecture of Alzheimer's disease: beyond APP, PSENs and APOE. Neurobiol Aging 33:437-456.

Haass C, Kaether C, Thinakaran G, Sisodia S (2012) Trafficking and Proteolytic Processing of APP. Cold Spring Harb Perspect Med 2:a006270.

Haass C, Koo EH, Capell A, Teplow DB, Selkoe DJ (1995a) Polarized sorting of beta-amyloid precursor protein and its proteolytic products in MDCK cells is regulated by two independent signals. J Cell Biol 128:537-547.

Haass C, Lemere CA, Capell A, Citron M, Seubert P, Schenk D, Lannfelt L, Selkoe DJ (1995b) The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. Nat Med 1:1291-1296.

Haass C, Selkoe DJ (1993) Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. Cell 75:1039-1042.

Hamaguchi T, Ono K, Murase A, Yamada M (2009) Phenolic compounds prevent Alzheimer's pathology through different effects on the amyloid-beta aggregation pathway. Am J Pathol 175:2557-2565.

Hamid R, Kilger E, Willem M, Vassallo N, Kostka M, Bornhovd C, Reichert AS, Kretzschmar HA, Haass C, Herms J (2007) Amyloid precursor protein intracellular domain modulates cellular calcium homeostasis and ATP content. J Neurochem 102:1264-1275.

Han BH, Holtzman DM (2000) BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. J Neurosci 20:5775-5781.

Hanger DP, Hughes K, Woodgett JR, Brion JP, Anderton BH (1992) Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. Neurosci Lett 147:58-62.

Haque AM, Hashimoto M, Katakura M, Hara Y, Shido O (2008) Green tea catechins prevent cognitive deficits caused by Abeta1-40 in rats. J Nutr Biochem 19:619-626.

Haque AM, Hashimoto M, Katakura M, Tanabe Y, Hara Y, Shido O (2006) Long-term administration of green tea catechins improves spatial cognition learning ability in rats. J Nutr 136:1043-1047.

Hardy J, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends in pharmacological sciences 12:383-388.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.

Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. Science 256:184-185.

Harel A, Wu F, Mattson MP, Morris CM, Yao PJ (2008) Evidence for CALM in directing VAMP2 trafficking. Traffic 9:417-429.

Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Williams A, Jones N, Thomas C, Stretton A, Morgan AR, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Morgan K, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Love S, Kehoe PG, Hardy J, Mead S, Fox N, Rossor M, Collinge J, Maier W, Jessen F, Schurmann B, Heun R, van den Bussche H, Heuser I, Kornhuber J, Wiltfang J, Dichgans M, Frolich L, Hampel H, Hull M, Rujescu D, Goate AM, Kauwe JS, Cruchaga C, Nowotny P, Morris JC, Mayo K, Sleegers K, Bettens K, Engelborghs S, De Deyn PP, Van Broeckhoven C, Livingston G, Bass NJ, Gurling H, McQuillin A, Gwilliam R, Deloukas P, Al-Chalabi A, Shaw CE, Tsolaki M, Singleton AB, Guerreiro R, Muhleisen TW, Nothen MM, Moebus S, Jockel KH, Klopp N, Wichmann HE, Carrasquillo MM, Pankratz VS, Younkin SG, Holmans PA, O'Donovan M, Owen MJ, Williams J (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet 41:1088-1093.

Harrison SM, Harper AJ, Hawkins J, Duddy G, Grau E, Pugh PL, Winter PH, Shilliam CS, Hughes ZA, Dawson LA, Gonzalez MI, Upton N, Pangalos MN, Dingwall C (2003) BACE1 (beta-secretase) transgenic and knockout mice: identification of neurochemical deficits and behavioral changes. Mol Cell Neurosci 24:646-655.

Hartman RE, Shah A, Fagan AM, Schwetye KE, Parsadanian M, Schulman RN, Finn MB, Holtzman DM (2006) Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease. Neurobiol Dis 24:506-515.

Hartmann D, De Strooper B, Saftig P (1999) Presenilin-1 deficiency leads to loss of Cajal-Retzius neurons and cortical dysplasia similar to human type 2 lissencephaly. Current biology : CB 9:719-727.

Hartmann D, de Strooper B, Serneels L, Craessaerts K, Herreman A, Annaert W, Umans L, Lubke T, Lena Illert A, von Figura K, Saftig P (2002) The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum Mol Genet 11:2615-2624.

He G, Luo W, Li P, Remmers C, Netzer WJ, Hendrick J, Bettayeb K, Flajolet M, Gorelick F, Wennogle LP, Greengard P (2010) Gamma-secretase activating protein is a therapeutic target for Alzheimer's disease. Nature 467:95-98.

He W, Shi Q, Hu X, Yan R (2007) The membrane topology of RTN3 and its effect on binding of RTN3 to BACE1. J Biol Chem 282:29144-29151.

He X, Li F, Chang WP, Tang J (2005) GGA proteins mediate the recycling pathway of memapsin 2 (BACE). J Biol Chem 280:11696-11703.

Heber S, Herms J, Gajic V, Hainfellner J, Aguzzi A, Rulicke T, von Kretzschmar H, von Koch C, Sisodia S, Tremml P, Lipp HP, Wolfer DP, Muller U (2000) Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. J Neurosci 20:7951-7963.

Hebert AE, Dash PK (2002) Extracellular signal-regulated kinase activity in the entorhinal cortex is necessary for long-term spatial memory. Learn Mem 9:156-166.

Heim KE, Tagliaferro AR, Bobilya DJ (2002) Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. Journal of Nutritional Biochemistry 13:572-584.

Hendriks L, van Duijn CM, Cras P, Cruts M, Van Hul W, van Harskamp F, Warren A, McInnis MG, Antonarakis SE, Martin JJ, et al. (1992) Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the beta-amyloid precursor protein gene. Nat Genet 1:218-221.

Herms J, Anliker B, Heber S, Ring S, Fuhrmann M, Kretzschmar H, Sisodia S, Muller U (2004) Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. EMBO J 23:4106-4115.

Herreman A, Hartmann D, Annaert W, Saftig P, Craessaerts K, Serneels L, Umans L, Schrijvers V, Checler F, Vanderstichele H, Baekelandt V, Dressel R, Cupers P, Huylebroeck D, Zwijsen A, Van Leuven F, De Strooper B (1999) Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc Natl Acad Sci U S A 96:11872-11877.

Herrmann N, Li A, Lanctot K (2011) Memantine in dementia: a review of the current evidence. Expert Opin Pharmacother 12:787-800.

Hetman M, Kanning K, Cavanaugh JE, Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem 274:22569-22580.

Ho CS, Marinescu V, Steinhilb ML, Gaut JR, Turner RS, Stuenkel EL (2002) Synergistic effects of Munc18a and X11 proteins on amyloid precursor protein metabolism. J Biol Chem 277:27021-27028.

Ho L, Ferruzzi MG, Janle EM, Wang J, Gong B, Chen TY, Lobo J, Cooper B, Wu QL, Talcott ST, Percival SS, Simon JE, Pasinetti GM (2013) Identification of brain-targeted bioactive dietary quercetin-3-O-glucuronide as a novel intervention for Alzheimer's disease. FASEB J 27:769-781.

Hoe HS, Cooper MJ, Burns MP, Lewis PA, van der Brug M, Chakraborty G, Cartagena CM, Pak DT, Cookson MR, Rebeck GW (2007) The metalloprotease inhibitor TIMP-3 regulates amyloid precursor protein and apolipoprotein E receptor proteolysis. J Neurosci 27:10895-10905.

Hoey SE, Buonocore F, Cox CJ, Hammond VJ, Perkinton MS, Williams RJ (2013) AMPA Receptor Activation Promotes Non-Amyloidogenic Amyloid Precursor Protein Processing and Suppresses Neuronal Amyloid-beta Production. PLoS ONE 8:e78155.

Hoey SE, Williams RJ, Perkinton MS (2009) Synaptic NMDA Receptor Activation Stimulates -Secretase Amyloid Precursor Protein Processing and Inhibits Amyloid- Production. Journal of Neuroscience 29:4442-4460.

Holtzman DM, Morris JC, Goate AM (2011) Alzheimer's disease: the challenge of the second century. Sci Transl Med 3:77sr71.

Hong L, Koelsch G, Lin X, Wu S, Terzyan S, Ghosh AK, Zhang XC, Tang J (2000) Structure of the protease domain of memapsin 2 (beta-secretase) complexed with inhibitor. Science 290:150-153.

Hook V, Toneff T, Bogyo M, Greenbaum D, Medzihradszky KF, Neveu J, Lane W, Hook G, Reisine T (2005) Inhibition of cathepsin B reduces beta-amyloid production in regulated secretory vesicles of neuronal chromaffin cells: evidence for cathepsin B as a candidate beta-secretase of Alzheimer's disease. Biological chemistry 386:931-940.

Hook VY, Kindy M, Hook G (2008) Inhibitors of cathepsin B improve memory and reduce beta-amyloid in transgenic Alzheimer disease mice expressing the wild-type, but not the Swedish mutant, beta-secretase site of the amyloid precursor protein. J Biol Chem 283:7745-7753.

Hook VY, Kindy M, Reinheckel T, Peters C, Hook G (2009) Genetic cathepsin B deficiency reduces beta-amyloid in transgenic mice expressing human wild-type amyloid precursor protein. Biochem Biophys Res Commun 386:284-288.

Hu J, Igarashi A, Kamata M, Nakagawa H (2001) Angiotensin-converting enzyme degrades Alzheimer amyloid beta-peptide (A beta ); retards A beta aggregation, deposition, fibril formation; and inhibits cytotoxicity. J Biol Chem 276:47863-47868.

Hu X, He W, Diaconu C, Tang X, Kidd GJ, Macklin WB, Trapp BD, Yan R (2008) Genetic deletion of BACE1 in mice affects remyelination of sciatic nerves. FASEB J 22:2970-2980.

Hu X, Hicks CW, He W, Wong P, Macklin WB, Trapp BD, Yan R (2006) Bace1 modulates myelination in the central and peripheral nervous system. Nat Neurosci 9:1520-1525.

Hu X, Zhou X, He W, Yang J, Xiong W, Wong P, Wilson CG, Yan R (2010) BACE1 deficiency causes altered neuronal activity and neurodegeneration. J Neurosci 30:8819-8829.

Hung AY, Haass C, Nitsch RM, Qiu WQ, Citron M, Wurtman RJ, Growdon JH, Selkoe DJ (1993) Activation of protein kinase C inhibits cellular production of the amyloid beta-protein. J Biol Chem 268:22959-22962.

Huse JT, Pijak DS, Leslie GJ, Lee VM, Doms RW (2000) Maturation and endosomal targeting of beta-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease beta-secretase. J Biol Chem 275:33729-33737.

Hussain I, Powell D, Howlett DR, Tew DG, Meek TD, Chapman C, Gloger IS, Murphy KE, Southan CD, Ryan DM, Smith TS, Simmons DL, Walsh FS, Dingwall C, Christie G (1999) Identification of a novel aspartic protease (Asp 2) as beta-secretase. Mol Cell Neurosci 14:419-427.

Hynd MR, Scott HL, Dodd PR (2004) Differential expression of N-methyl-D-aspartate receptor NR2 isoforms in Alzheimer's disease. J Neurochem 90:913-919.

Illenberger S, Zheng-Fischhofer Q, Preuss U, Stamer K, Baumann K, Trinczek B, Biernat J, Godemann R, Mandelkow EM, Mandelkow E (1998) The endogenous and cell cycle-dependent phosphorylation of tau protein in living cells: implications for Alzheimer's disease. Mol Biol Cell 9:1495-1512.

Imbimbo BP, Panza F, Frisardi V, Solfrizzi V, D'Onofrio G, Logroscino G, Seripa D, Pilotto A (2011) Therapeutic intervention for Alzheimer's disease with gamma-secretase inhibitors: still a viable option? Expert opinion on investigational drugs 20:325-341.

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR (1998) Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. Neuron 21:869-883.

Inomata H, Nakamura Y, Hayakawa A, Takata H, Suzuki T, Miyazawa K, Kitamura N (2003) A scaffold protein JIP-1b enhances amyloid precursor protein phosphorylation by JNK and its association with kinesin light chain 1. J Biol Chem 278:22946-22955.

Inoue E, Deguchi-Tawarada M, Togawa A, Matsui C, Arita K, Katahira-Tayama S, Sato T, Yamauchi E, Oda Y, Takai Y (2009) Synaptic activity prompts gamma-

secretase-mediated cleavage of EphA4 and dendritic spine formation. J Cell Biol 185:551-564.

INSERM Collective Expertise Centre (2007) Alzheimer's Disease: Scientific, medical and societal implications. Paris: Institut national de la sante et de la recherche medicale.

Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee HJ, Saido TC (2001) Metabolic regulation of brain Abeta by neprilysin. Science 292:1550-1552.

Jacobsen KT, Iverfeldt K (2009) Amyloid precursor protein and its homologues: a family of proteolysis-dependent receptors. Cellular and molecular life sciences : CMLS 66:2299-2318.

Jang SW, Liu X, Yepes M, Shepherd KR, Miller GW, Liu Y, Wilson WD, Xiao G, Blanchi B, Sun YE, Ye KQ (2010) A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. P Natl Acad Sci USA 107:2687-2692.

Johnson KA, Fox NC, Sperling RA, Klunk WE (2012) Brain imaging in Alzheimer disease. Cold Spring Harb Perspect Med 2:a006213.

Jonsson T, Atwal JK, Steinberg S, Snaedal J, Jonsson PV, Bjornsson S, Stefansson H, Sulem P, Gudbjartsson D, Maloney J, Hoyte K, Gustafson A, Liu Y, Lu Y, Bhangale T, Graham RR, Huttenlocher J, Bjornsdottir G, Andreassen OA, Jonsson EG, Palotie A, Behrens TW, Magnusson OT, Kong A, Thorsteinsdottir U, Watts RJ, Stefansson K (2012) A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. Nature 488:96-99.

Jorissen E, Prox J, Bernreuther C, Weber S, Schwanbeck R, Serneels L, Snellinx A, Craessaerts K, Thathiah A, Tesseur I, Bartsch U, Weskamp G, Blobel CP, Glatzel M, De Strooper B, Saftig P (2010) The disintegrin/metalloproteinase ADAM10 is essential for the establishment of the brain cortex. J Neurosci 30:4833-4844.

Joseph JA, Shukitt-Hale B, Denisova NA, Bielinski D, Martin A, McEwen JJ, Bickford PC (1999) Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J Neurosci 19:8114-8121.

Jovanovic S, Steenken S, Simic M, Hara Y (1998) Flavonoids in Health and Disease. New York: Marcel Dekker.

Jurisch-Yaksi N, Sannerud R, Annaert W (2013) A fast growing spectrum of biological functions of gamma-secretase in development and disease. Biochim Biophys Acta 1828:2815-2827.

Kaether C, Haass C, Steiner H (2006) Assembly, trafficking and function of gamma-secretase. Neuro-degenerative diseases 3:275-283.

Kalvodova L, Kahya N, Schwille P, Ehehalt R, Verkade P, Drechsel D, Simons K (2005) Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro. J Biol Chem 280:36815-36823.

Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, Sisodia S, Malinow R (2003) APP processing and synaptic function. Neuron 37:925-937.

Kanatsu K, Morohashi Y, Suzuki M, Kuroda H, Watanabe T, Tomita T, Iwatsubo T (2014) Decreased CALM expression reduces Abeta42 to total Abeta ratio through clathrin-mediated endocytosis of gamma-secretase. Nat Commun 5:3386.

Kapogiannis D, Mattson MP (2011) Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease. Lancet Neurol 10:187-198.

Karkkainen I, Rybnikova E, Pelto-Huikko M, Huovila AP (2000) Metalloprotease-disintegrin (ADAM) genes are widely and differentially expressed in the adult CNS. Mol Cell Neurosci 15:547-560.

Karlinsky H, Vaula G, Haines JL, Ridgley J, Bergeron C, Mortilla M, Tupler RG, Percy ME, Robitaille Y, Noldy NE, et al. (1992) Molecular and prospective phenotypic characterization of a pedigree with familial Alzheimer's disease and a missense mutation in codon 717 of the beta-amyloid precursor protein gene. Neurology 42:1445-1453.

Kaufman AM, Milnerwood AJ, Sepers MD, Coquinco A, She K, Wang L, Lee H, Craig AM, Cynader M, Raymond LA (2012) Opposing roles of synaptic and extrasynaptic NMDA receptor signaling in cocultured striatal and cortical neurons. J Neurosci 32:3992-4003.

Kawahata I, Yoshida M, Sun W, Nakajima A, Lai Y, Osaka N, Matsuzaki K, Yokosuka A, Mimaki Y, Naganuma A, Tomioka Y, Yamakuni T (2013) Potent activity of nobiletin-rich Citrus reticulata peel extract to facilitate cAMP/PKA/ERK/CREB signaling associated with learning and memory in cultured hippocampal neurons: identification of the substances responsible for the pharmacological action. J Neural Transm 120:1397-1409.

Kelly A, Laroche S, Davis S (2003) Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase in hippocampal circuitry is required for consolidation and reconsolidation of recognition memory. J Neurosci 23:5354-5360.

Kepe V, Barrio JR, Huang SC, Ercoli L, Siddarth P, Shoghi-Jadid K, Cole GM, Satyamurthy N, Cummings JL, Small GW, Phelps ME (2006) Serotonin 1A receptors in the living brain of Alzheimer's disease patients. Proc Natl Acad Sci U S A 103:702-707.

Khan UA, Liu L, Provenzano FA, Berman DE, Profaci CP, Sloan R, Mayeux R, Duff KE, Small SA (2014) Molecular drivers and cortical spread of lateral entorhinal

cortex dysfunction in preclinical Alzheimer's disease. Nat Neurosci 17:304-311.

Kibbey MC, Jucker M, Weeks BS, Neve RL, Van Nostrand WE, Kleinman HK (1993) beta-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin. Proc Natl Acad Sci U S A 90:10150-10153.

Kim DY, Carey BW, Wang H, Ingano LA, Binshtok AM, Wertz MH, Pettingell WH, He P, Lee VM, Woolf CJ, Kovacs DM (2007) BACE1 regulates voltage-gated sodium channels and neuronal activity. Nat Cell Biol 9:755-764.

Kim H, Park BS, Lee KG, Choi CY, Jang SS, Kim YH, Lee SE (2005a) Effects of naturally occurring compounds on fibril formation and oxidative stress of beta-amyloid. Journal of agricultural and food chemistry 53:8537-8541.

Kim JA, Kim HL (2001) Cell-free expression and functional reconstitution of CALM in clathrin assembly. Experimental & molecular medicine 33:89-94.

Kim M, Suh J, Romano D, Truong MH, Mullin K, Hooli B, Norton D, Tesco G, Elliott K, Wagner SL, Moir RD, Becker KD, Tanzi RE (2009) Potential late-onset Alzheimer's disease-associated mutations in the ADAM10 gene attenuate {alpha}-secretase activity. Hum Mol Genet 18:3987-3996.

Kim MJ, Dunah AW, Wang YT, Sheng M (2005b) Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46:745-760.

Kimberly WT, Xia W, Rahmati T, Wolfe MS, Selkoe DJ (2000) The transmembrane aspartates in presenilin 1 and 2 are obligatory for gamma-secretase activity and amyloid beta-protein generation. J Biol Chem 275:3173-3178.

Kimberly WT, Zheng JB, Guenette SY, Selkoe DJ (2001) The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. J Biol Chem 276:40288-40292.

King GD, Scott Turner R (2004) Adaptor protein interactions: modulators of amyloid precursor protein metabolism and Alzheimer's disease risk? Experimental neurology 185:208-219.

Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT (2003) Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. J Cell Sci 116:3339-3346.

Kitaguchi N, Takahashi Y, Tokushima Y, Shiojiri S, Ito H (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. Nature 331:530-532.

Klafki HW, Lewczuk P, Kamrowski-Kruck H, Maler JM, Muller K, Peters O, Heuser I, Jessen F, Popp J, Frolich L, Wolf S, Prinz B, Luckhaus C, Schroder J, Pantel J, Gertz HJ, Kolsch H, Muller BW, Esselmann H, Bibl M, Kornhuber J, Wiltfang J (2009) Measurement of ERK 1/2 in CSF from patients with neuropsychiatric

disorders and evidence for the presence of the activated form. J Alzheimers Dis 18:613-622.

Kojro E, Postina R, Buro C, Meiringer C, Gehrig-Burger K, Fahrenholz F (2006) The neuropeptide PACAP promotes the alpha-secretase pathway for processing the Alzheimer amyloid precursor protein. FASEB J 20:512-514.

Koo EH, Squazzo SL (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J Biol Chem 269:17386-17389.

Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137:216-233.

Kuhn P-H, Wang H, Dislich B, Colombo A, Zeitschel U, Ellwart JW, Kremmer E, Roßner S, Lichtenthaler SF (2010) ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons. The EMBO Journal 29:3020-3032.

Kumar-Singh S, Dewachter I, Moechars D, Lubke U, De Jonghe C, Ceuterick C, Checler F, Naidu A, Cordell B, Cras P, Van Broeckhoven C, Van Leuven F (2000) Behavioral disturbances without amyloid deposits in mice overexpressing human amyloid precursor protein with Flemish (A692G) or Dutch (E693Q) mutation. Neurobiol Dis 7:9-22.

Kumar-Singh S, Theuns J, Van Broeck B, Pirici D, Vennekens K, Corsmit E, Cruts M, Dermaut B, Wang R, Van Broeckhoven C (2006) Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. Hum Mutat 27:686-695.

Kuriyama S, Hozawa A, Ohmori K, Shimazu T, Matsui T, Ebihara S, Awata S, Nagatomi R, Arai H, Tsuji I (2006) Green tea consumption and cognitive function: a cross-sectional study from the Tsurugaya Project 1. Am J Clin Nutr 83:355-361.

Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, Wen H, Chiang HC, Xu G, Koliatsos VE, Borchelt DR, Price DL, Lee HK, Wong PC (2005) BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. J Neurosci 25:11693-11709.

Lambert JC, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, Combarros O, Zelenika D, Bullido MJ, Tavernier B, Letenneur L, Bettens K, Berr C, Pasquier F, Fievet N, Barberger-Gateau P, Engelborghs S, De Deyn P, Mateo I, Franck A, Helisalmi S, Porcellini E, Hanon O, de Pancorbo MM, Lendon C, Dufouil C, Jaillard C, Leveillard T, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossu P, Piccardi P, Annoni G, Seripa D, Galimberti D, Hannequin D, Licastro F, Soininen H, Ritchie K, Blanche H, Dartigues JF, Tzourio C, Gut I, Van Broeckhoven C, Alperovitch A, Lathrop M, Amouyel P (2009) Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nat Genet 41:1094-1099.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 95:6448-6453.

Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc Natl Acad Sci U S A 96:3922-3927.

Larner AJ (2013) Presenilin-1 mutations in Alzheimer's disease: an update on genotype-phenotype relationships. J Alzheimers Dis 37:653-659.

Laurin D, Masaki KH, Foley DJ, White LR, Launer LJ (2004) Midlife dietary intake of antioxidants and risk of late-life incident dementia: the Honolulu-Asia Aging Study. Am J Epidemiol 159:959-967.

Lavado A, Oliver G (2014) Jagged1 is necessary for postnatal and adult neurogenesis in the dentate gyrus. Dev Biol.

Lazarov VK, Fraering PC, Ye W, Wolfe MS, Selkoe DJ, Li H (2006) Electron microscopic structure of purified, active gamma-secretase reveals an aqueous intramembrane chamber and two pores. Proc Natl Acad Sci U S A 103:6889-6894.

Lee MS, Kao SC, Lemere CA, Xia W, Tseng HC, Zhou Y, Neve R, Ahlijanian MK, Tsai LH (2003) APP processing is regulated by cytoplasmic phosphorylation. J Cell Biol 163:83-95.

Lemberg MK (2011) Intramembrane proteolysis in regulated protein trafficking. Traffic 12:1109-1118.

Lemberg MK, Martoglio B (2002) Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. Molecular cell 10:735-744.

Lemere CA, Masliah E (2010) Can Alzheimer disease be prevented by amyloid-beta immunotherapy? Nat Rev Neurol 6:108-119.

Lesne S, Ali C, Gabriel C, Croci N, MacKenzie ET, Glabe CG, Plotkine M, Marchand-Verrecchia C, Vivien D, Buisson A (2005) NMDA receptor activation inhibits alpha-secretase and promotes neuronal amyloid-beta production. J Neurosci 25:9367-9377.

Lesné S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006) A specific amyloid-β protein assembly in the brain impairs memory. Nature 440:352-357.

Letenneur L, Proust-Lima C, Le Gouge A, Dartigues J, Barberger-Gateau P (2007) Flavonoid Intake and Cognitive Decline over a 10-Year Period. American Journal of Epidemiology 165:1364-1371.

Levites Y, Amit T, Mandel S, Youdim MB (2003) Neuroprotection and neurorescue against Abeta toxicity and PKC-dependent release of nonamyloidogenic soluble precursor protein by green tea polyphenol (-)-epigallocatechin-3-gallate. FASEB J 17:952-954.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, Wang K, et al. (1995a) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 269:973-977.

Levy-Lahad E, Wijsman EM, Nemens E, Anderson L, Goddard KA, Weber JL, Bird TD, Schellenberg GD (1995b) A familial Alzheimer's disease locus on chromosome 1. Science 269:970-973.

Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 293:1487-1491.

Li D, Parks SB, Kushner JD, Nauman D, Burgess D, Ludwigsen S, Partain J, Nixon RR, Allen CN, Irwin RP, Jakobs PM, Litt M, Hershberger RE (2006) Mutations of presenilin genes in dilated cardiomyopathy and heart failure. Am J Hum Genet 79:1030-1039.

Li H, Wang B, Wang Z, Guo Q, Tabuchi K, Hammer RE, Sudhof TC, Zheng H (2010) Soluble amyloid precursor protein (APP) regulates transthyretin and Klotho gene expression without rescuing the essential function of APP. Proc Natl Acad Sci U S A 107:17362-17367.

Li S, Hong S, Shepardson NE, Walsh DM, Shankar GM, Selkoe D (2009) Soluble Oligomers of Amyloid β Protein Facilitate Hippocampal Long-Term Depression by Disrupting Neuronal Glutamate Uptake. Neuron 62:788-801.

Li S, Jin M, Koeglsperger T, Shepardson NE, Shankar GM, Selkoe DJ (2011) Soluble A Oligomers Inhibit Long-Term Potentiation through a Mechanism Involving Excessive Activation of Extrasynaptic NR2B-Containing NMDA Receptors. Journal of Neuroscience 31:6627-6638.

Li YM, Lai MT, Xu M, Huang Q, DiMuzio-Mower J, Sardana MK, Shi XP, Yin KC, Shafer JA, Gardell SJ (2000) Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. Proc Natl Acad Sci U S A 97:6138-6143.

Lichtenthaler SF (2011) Alpha-secretase in Alzheimer's disease: molecular identity, regulation and therapeutic potential. J Neurochem 116:10-21.

Lichtenthaler SF, Haass C, Steiner H (2011) Regulated intramembrane proteolysis - lessons from amyloid precursor protein processing. Journal of Neurochemistry 117:779-796.

Lin C, Chen T, Chiu M, Way T, Lin J (2009) Epigallocatechin gallate (EGCG) suppresses β-amyloid-induced neurotoxicity through inhibiting c-Abl/FE65 nuclear translocation and GSK3β activation. Neurobiology of Aging 30:81-92.

Lin X, Koelsch G, Wu S, Downs D, Dashti A, Tang J (2000) Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein. Proc Natl Acad Sci U S A 97:1456-1460.

Liu CC, Kanekiyo T, Xu H, Bu G (2013) Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol 9:106-118.

Llorens F, Garcia L, Itarte E, Gomez N (2002) Apigenin and LY294002 prolong EGF-stimulated ERK1/2 activation in PC12 cells but are unable to induce full differentiation. FEBS Lett 510:149-153.

Loon YH, Wong JW, Yap SP, Yuen KH (2005) Determination of flavonoids from Orthosiphon stamineus in plasma using a simple HPLC method with ultraviolet detection. Journal of chromatography B, Analytical technologies in the biomedical and life sciences 816:161-166.

Lu B, Martinowich K (2008) Cell biology of BDNF and its relevance to schizophrenia. Novartis Foundation symposium 289:119-129; discussion 129-135, 193-115.

Lu C, Wang Y, Furukawa K, Fu W, Ouyang X, Mattson MP (2006) Evidence that caspase-1 is a negative regulator of AMPA receptor-mediated long-term potentiation at hippocampal synapses. J Neurochem 97:1104-1110.

Luo Y, Bolon B, Kahn S, Bennett BD, Babu-Khan S, Denis P, Fan W, Kha H, Zhang J, Gong Y, Martin L, Louis JC, Yan Q, Richards WG, Citron M, Vassar R (2001) Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. Nat Neurosci 4:231-232.

Madeira A, Pommet JM, Prochiantz A, Allinquant B (2005) SET protein (TAF1beta, I2PP2A) is involved in neuronal apoptosis induced by an amyloid precursor protein cytoplasmic subdomain. FASEB J 19:1905-1907.

Maher P, Akaishi T, Abe K (2006) Flavonoid fisetin promotes ERK-dependent long-term potentiation and enhances memory. Proceedings of the National Academy of Sciences 103:16568-16573.

Maher P, Salgado KF, Zivin JA, Lapchak PA (2007) A novel approach to screening for new neuroprotective compounds for the treatment of stroke. Brain Res 1173:117-125.

Malinverno M, Carta M, Epis R, Marcello E, Verpelli C, Cattabeni F, Sala C, Mulle C, Di Luca M, Gardoni F (2010) Synaptic localization and activity of ADAM10 regulate excitatory synapses through N-cadherin cleavage. J Neurosci 30:16343-16355.

Manach (2004) Polyphenols: food sources and bioavailability. American Journal of Clinical Nutrition.

Mandelkow EM, Mandelkow E (2012) Biochemistry and cell biology of tau protein in neurofibrillary degeneration. Cold Spring Harb Perspect Med 2:a006247.

Mandelkow EM, Thies E, Trinczek B, Biernat J, Mandelkow E (2004) MARK/PAR1 kinase is a regulator of microtubule-dependent transport in axons. J Cell Biol 167:99-110.

Mann GE, Rowlands DJ, Li FY, de Winter P, Siow RC (2007) Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression. Cardiovascular research 75:261-274.

Marcello E, Gardoni F, Mauceri D, Romorini S, Jeromin A, Epis R, Borroni B, Cattabeni F, Sala C, Padovani A, Di Luca M (2007) Synapse-associated protein-97 mediates alpha-secretase ADAM10 trafficking and promotes its activity. J Neurosci 27:1682-1691.

Marcinkiewicz M, Seidah NG (2000) Coordinated expression of beta-amyloid precursor protein and the putative beta-secretase BACE and alpha-secretase ADAM10 in mouse and human brain. J Neurochem 75:2133-2143.

Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, de Strooper B, Hartmann D, Saftig P (2005) ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. Proc Natl Acad Sci U S A 102:9182-9187.

Markesbery WR (1997) Oxidative stress hypothesis in Alzheimer's disease. Free Radic Biol Med 23:134-147.

Marquer C, Devauges V, Cossec JC, Liot G, Lecart S, Saudou F, Duyckaerts C, Leveque-Fort S, Potier MC (2011) Local cholesterol increase triggers amyloid precursor protein-Bace1 clustering in lipid rafts and rapid endocytosis. FASEB J 25:1295-1305.

Marzolo MP, Bu G (2009) Lipoprotein receptors and cholesterol in APP trafficking and proteolytic processing, implications for Alzheimer's disease. Semin Cell Dev Biol 20:191-200.

Maskos K, Fernandez-Catalan C, Huber R, Bourenkov GP, Bartunik H, Ellestad GA, Reddy P, Wolfson MF, Rauch CT, Castner BJ, Davis R, Clarke HR, Petersen M, Fitzner JN, Cerretti DP, March CJ, Paxton RJ, Black RA, Bode W (1998) Crystal structure of the catalytic domain of human tumor necrosis factor-alpha-converting enzyme. Proc Natl Acad Sci U S A 95:3408-3412.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A 82:4245-4249.

Mattson MP (2003) Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. Neuromolecular medicine 3:65-94.

Mattson MP, Barger SW, Cheng B, Lieberburg I, Smith-Swintosky VL, Rydel RE (1993) beta-Amyloid precursor protein metabolites and loss of neuronal Ca2+ homeostasis in Alzheimer's disease. Trends Neurosci 16:409-414.

May PC, Lampert-Etchells M, Johnson SA, Poirier J, Masters JN, Finch CE (1990) Dynamics of gene expression for a hippocampal glycoprotein elevated in Alzheimer's disease and in response to experimental lesions in rat. Neuron 5:831-839.

Mazzitelli S, Xu P, Ferrer I, Davis RJ, Tournier C (2011) The Loss of c-Jun N-Terminal Protein Kinase Activity Prevents the Amyloidogenic Cleavage of Amyloid Precursor Protein and the Formation of Amyloid Plaques In Vivo. J Neurosci 31:16969-16976.

McDermott JR, Gibson AM (1991) The processing of Alzheimer A4/beta-amyloid protein precursor: identification of a human brain metallopeptidase which cleaves -Lys-Leu- in a model peptide. Biochem Biophys Res Commun 179:1148-1154.

McGeer EG, McGeer PL (2001) Innate immunity in Alzheimer's disease: a model for local inflammatory reactions. Molecular interventions 1:22-29.

McGeer PL, Akiyama H, Itagaki S, McGeer EG (1989) Activation of the classical complement pathway in brain tissue of Alzheimer patients. Neurosci Lett 107:341-346.

McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7:263-269.

McLoughlin DM, Miller CC (1996) The intracellular cytoplasmic domain of the Alzheimer's disease amyloid precursor protein interacts with phosphotyrosine-binding domain proteins in the yeast two-hybrid system. FEBS Lett 397:197-200.

McLoughlin DM, Miller CC (2008) The FE65 proteins and Alzheimer's disease. J Neurosci Res 86:744-754.

Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M, de Calignon A, Rozkalne A, Koenigsknecht-Talboo J, Holtzman DM, Bacskai BJ, Hyman BT (2008) Rapid appearance and local toxicity of amyloid-beta plaques in a mouse model of Alzheimer's disease. Nature 451:720-724.

Mignet N, Seguin J, Ramos Romano M, Brulle L, Touil YS, Scherman D, Bessodes M, Chabot GG (2012) Development of a liposomal formulation of the natural flavonoid fisetin. International journal of pharmaceutics 423:69-76.

Miller CC, McLoughlin DM, Lau KF, Tennant ME, Rogelj B (2006) The X11 proteins, Abeta production and Alzheimer's disease. Trends Neurosci 29:280-285.

Miller S, Yasuda M, Coats JK, Jones Y, Martone ME, Mayford M (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. Neuron 36:507-519.

Mills J, Reiner PB (1999) Mitogen-activated protein kinase is involved in N-methyl-D-aspartate receptor regulation of amyloid precursor protein cleavage. Neuroscience 94:1333-1338.

Minano-Molina AJ, Espana J, Martin E, Barneda-Zahonero B, Fado R, Sole M, Trullas R, Saura CA, Rodriguez-Alvarez J (2011) Soluble Oligomers of Amyloid- Peptide Disrupt Membrane Trafficking of -Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptor Contributing to Early Synapse Dysfunction. Journal of Biological Chemistry 286:27311-27321.

Morales-Corraliza J, Mazzella MJ, Berger JD, Diaz NS, Choi JH, Levy E, Matsuoka Y, Planel E, Mathews PM (2009) In vivo turnover of tau and APP metabolites in the brains of wild-type and Tg2576 mice: greater stability of sAPP in the beta-amyloid depositing mice. PLoS ONE 4:e7134.

Mori T, Rezai-Zadeh K, Koyama N, Arendash GW, Yamaguchi H, Kakuda N, Horikoshi-Sakuraba Y, Tan J, Town T (2012) Tannic acid is a natural beta-secretase inhibitor that prevents cognitive impairment and mitigates Alzheimer-like pathology in transgenic mice. J Biol Chem 287:6912-6927.

Mowrer KR, Wolfe MS (2008) Promotion of BACE1 mRNA alternative splicing reduces amyloid beta-peptide production. J Biol Chem 283:18694-18701.

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1:345-347.

Muller U, Cristina N, Li ZW, Wolfer DP, Lipp HP, Rulicke T, Brandner S, Aguzzi A, Weissmann C (1994) Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. Cell 79:755-765.

Muller UC, Zheng H (2012) Physiological functions of APP family proteins. Cold Spring Harb Perspect Med 2:a006288.

Muraguchi T, Takegami Y, Ohtsuka T, Kitajima S, Chandana EP, Omura A, Miki T, Takahashi R, Matsumoto N, Ludwig A, Noda M, Takahashi C (2007) RECK modulates Notch signaling during cortical neurogenesis by regulating ADAM10 activity. Nat Neurosci 10:838-845.

Murrell J, Farlow M, Ghetti B, Benson MD (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science 254:97-99.

Naruse S, Igarashi S, Kobayashi H, Aoki K, Inuzuka T, Kaneko K, Shimizu T, Iihara K, Kojima T, Miyatake T, et al. (1991) Mis-sense mutation Val----Ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease. Lancet 337:978-979.

Ni CY, Murphy MP, Golde TE, Carpenter G (2001) gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science 294:2179-2181.

Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M (2009) APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature 457:981-989.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Naslund J, Lannfelt L (2001) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. Nat Neurosci 4:887-893.

Ninomiya H, Roch JM, Sundsmo MP, Otero DA, Saitoh T (1993) Amino acid sequence RERMS represents the active domain of amyloid beta/A4 protein precursor that promotes fibroblast growth. J Cell Biol 121:879-886.

Nunomura A, Perry G, Pappolla MA, Friedland RP, Hirai K, Chiba S, Smith MA (2000) Neuronal oxidative stress precedes amyloid-beta deposition in Down syndrome. J Neuropathol Exp Neurol 59:1011-1017.

Nuutinen T, Suuronen T, Kauppinen A, Salminen A (2009) Clusterin: a forgotten player in Alzheimer's disease. Brain research reviews 61:89-104.

Obregon DF, Rezai-Zadeh K, Bai Y, Sun N, Hou H, Ehrhart J, Zeng J, Mori T, Arendash GW, Shytle D, Town T, Tan J (2006) ADAM10 activation is required for green tea (-)-epigallocatechin-3-gallate-induced alpha-secretase cleavage of amyloid precursor protein. J Biol Chem 281:16419-16427.

Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA, Finch CE (1994) Purification and characterization of brain clusterin. Biochem Biophys Res Commun 204:1131-1136.

Ohno M, Sametsky EA, Younkin LH, Oakley H, Younkin SG, Citron M, Vassar R, Disterhoft JF (2004) BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. Neuron 41:27-33.

Ono K, Yoshiike Y, Takashima A, Hasegawa K, Naiki H, Yamada M (2003) Potent anti-amyloidogenic and fibril-destabilizing effects of polyphenols in vitro: implications for the prevention and therapeutics of Alzheimer's disease. J Neurochem 87:172-181.

Onozuka H, Nakajima A, Matsuzaki K, Shin RW, Ogino K, Saigusa D, Tetsu N, Yokosuka A, Sashida Y, Mimaki Y, Yamakuni T, Ohizumi Y (2008) Nobiletin, a citrus flavonoid, improves memory impairment and Abeta pathology in a transgenic mouse model of Alzheimer's disease. J Pharmacol Exp Ther 326:739-744.

Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S, Michel BF, Boada M, Frank A, Hock C (2003) Subacute

meningoencephalitis in a subset of patients with AD after Abeta42 immunization. Neurology 61:46-54.

Oyama F, Sawamura N, Kobayashi K, Morishima-Kawashima M, Kuramochi T, Ito M, Tomita T, Maruyama K, Saido TC, Iwatsubo T, Capell A, Walter J, Grunberg J, Ueyama Y, Haass C, Ihara Y (1998) Mutant presenilin 2 transgenic mouse: effect on an age-dependent increase of amyloid beta-protein 42 in the brain. J Neurochem 71:313-322.

Peiretti F, Deprez-Beauclair P, Bonardo B, Aubert H, Juhan-Vague I, Nalbone G (2003) Identification of SAP97 as an intracellular binding partner of TACE. J Cell Sci 116:1949-1957.

Perkinton MS, Standen CL, Lau KF, Kesavapany S, Byers HL, Ward M, McLoughlin DM, Miller CC (2004) The c-Abl tyrosine kinase phosphorylates the Fe65 adaptor protein to stimulate Fe65/amyloid precursor protein nuclear signaling. J Biol Chem 279:22084-22091.

Perry G, Roder H, Nunomura A, Takeda A, Friedlich AL, Zhu X, Raina AK, Holbrook N, Siedlak SL, Harris PL, Smith MA (1999) Activation of neuronal extracellular receptor kinase (ERK) in Alzheimer disease links oxidative stress to abnormal phosphorylation. Neuroreport 10:2411-2415.

Perry SW, Norman JP, Litzburg A, Gelbard HA (2004) Antioxidants are required during the early critical period, but not later, for neuronal survival. J Neurosci Res 78:485-492.

Petersen RB, Nunomura A, Lee HG, Casadesus G, Perry G, Smith MA, Zhu X (2007) Signal transduction cascades associated with oxidative stress in Alzheimer's disease. J Alzheimers Dis 11:143-152.

Pietta PG (2000) Flavonoids as antioxidants. Journal of natural products 63:1035-1042.

Ponte P, Gonzalez-DeWhitt P, Schilling J, Miller J, Hsu D, Greenberg B, Davis K, Wallace W, Lieberburg I, Fuller F (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. Nature 331:525-527.

Postina R, Schroeder A, Dewachter I, Bohl J, Schmitt U, Kojro E, Prinzen C, Endres K, Hiemke C, Blessing M, Flamez P, Dequenne A, Godaux E, van Leuven F, Fahrenholz F (2004) A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model. J Clin Invest 113:1456-1464.

Puzzo D, Privitera L, Leznik E, Fa M, Staniszewski A, Palmeri A, Arancio O (2008) Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. J Neurosci 28:14537-14545.

Quintero-Monzon O, Martin MM, Fernandez MA, Cappello CA, Krzysiak AJ, Osenkowski P, Wolfe MS (2011) Dissociation between the Processivity and Total Activity of gamma-Secretase: Implications for the Mechanism of

Alzheimer's Disease-Causing Presenilin Mutations. Biochemistry 50:9023-9035.

Rainey-Smith S, Schroetke LW, Bahia P, Fahmi A, Skilton R, Spencer JP, Rice-Evans C, Rattray M, Williams RJ (2008) Neuroprotective effects of hesperetin in mouse primary neurones are independent of CREB activation. Neurosci Lett 438:29-33.

Ramassamy C (2006) Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: A review of their intracellular targets. European Journal of Pharmacology 545:51-64.

Rapoport M, Dawson HN, Binder LI, Vitek MP, Ferreira A (2002) Tau is essential to beta -amyloid-induced neurotoxicity. Proc Natl Acad Sci U S A 99:6364-6369.

Raskind MA, Peskind ER, Wessel T, Yuan W (2000) Galantamine in AD: A 6-month randomized, placebo-controlled trial with a 6-month extension. The Galantamine USA-1 Study Group. Neurology 54:2261-2268.

Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH, Fraga CG (2000) Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. J Nutr 130:2109S-2114S.

Reinhard C, Hebert SS, De Strooper B (2005) The amyloid-beta precursor protein: integrating structure with biological function. EMBO J 24:3996-4006.

Reiss K, Maretzky T, Ludwig A, Tousseyn T, de Strooper B, Hartmann D, Saftig P (2005) ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. EMBO J 24:742-752.

Renouf M, Marmet C, Guy PA, Beaumont M, Lepage M, Williamson G, Dionisi F (2013) Dose-response plasma appearance of green tea catechins in adults. Molecular nutrition & food research 57:833-839.

Rezai-Zadeh K (2005) Green Tea Epigallocatechin-3-Gallate (EGCG) Modulates Amyloid Precursor Protein Cleavage and Reduces Cerebral Amyloidosis in Alzheimer Transgenic Mice. Journal of Neuroscience 25:8807-8814.

Rezai-Zadeh K, Arendash GW, Hou H, Fernandez F, Jensen M, Runfeldt M, Shytle RD, Tan J (2008) Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice. Brain Res 1214:177-187.

Rezai-Zadeh K, Douglas Shytle R, Bai Y, Tian J, Hou H, Mori T, Zeng J, Obregon D, Town T, Tan J (2009) Flavonoid-mediated presenilin-1 phosphorylation reduces Alzheimer's disease beta-amyloid production. J Cell Mol Med 13:574-588.

Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20:933-956.

RiceEvans C, Miller N (1997) Measurement of the antioxidant status of dietary constituents, low density lipoproteins and plasma. Prostag Leukotr Ess 57:499-505.

Richelle M, Tavazzi I, Enslen M, Offord EA (1999) Plasma kinetics in man of epicatechin from black chocolate. Eur J Clin Nutr 53:22-26.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19:4337-4348.

Roberson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, Mucke L (2007) Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. Science 316:750-754.

Roberts SB, Ripellino JA, Ingalls KM, Robakis NK, Felsenstein KM (1994) Non-amyloidogenic cleavage of the beta-amyloid precursor protein by an integral membrane metalloendopeptidase. J Biol Chem 269:3111-3116.

Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y, Chi H, Lin C, Holman K, Tsuda T, et al. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 376:775-778.

Rogelj B, Mitchell JC, Miller CC, McLoughlin DM (2006) The X11/Mint family of adaptor proteins. Brain research reviews 52:305-315.

Rogers SL, Friedhoff LT (1996) The efficacy and safety of donepezil in patients with Alzheimer's disease: results of a US Multicentre, Randomized, Double-Blind, Placebo-Controlled Trial. The Donepezil Study Group. Dementia 7:293-303.

Roghani M, Niknam A, Jalali-Nadoushan MR, Kiasalari Z, Khalili M, Baluchnejadmojarad T (2010) Oral pelargonidin exerts dose-dependent neuroprotection in 6-hydroxydopamine rat model of hemi-parkinsonism. Brain research bulletin 82:279-283.

Rohe M, Synowitz M, Glass R, Paul SM, Nykjaer A, Willnow TE (2009) Brain-derived neurotrophic factor reduces amyloidogenic processing through control of SORLA gene expression. J Neurosci 29:15472-15478.

Rosen LB, Ginty DD, Weber MJ, Greenberg ME (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 12:1207-1221.

Roskoski R, Jr. (2012) MEK1/2 dual-specificity protein kinases: structure and regulation. Biochem Biophys Res Commun 417:5-10.

Rosler M, Anand R, Cicin-Sain A, Gauthier S, Agid Y, Dal-Bianco P, Stahelin HB, Hartman R, Gharabawi M (1999) Efficacy and safety of rivastigmine in

patients with Alzheimer's disease: international randomised controlled trial. BMJ 318:633-638.

Saftig P, Reiss K (2011) The "A Disintegrin And Metalloproteases" ADAM10 and ADAM17: novel drug targets with therapeutic potential? European journal of cell biology 90:527-535.

Sagara Y, Vanhnasy J, Maher P (2004) Induction of PC12 cell differentiation by flavonoids is dependent upon extracellular signal-regulated kinase activation. J Neurochem 90:1144-1155.

Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, Hartmann D, Saftig P, Blobel CP (2004) Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol 164:769-779.

Sambamurti K, Kinsey R, Maloney B, Ge YW, Lahiri DK (2004) Gene structure and organization of the human beta-secretase (BACE) promoter. FASEB J 18:1034-1036.

Sanan DA, Weisgraber KH, Russell SJ, Mahley RW, Huang D, Saunders A, Schmechel D, Wisniewski T, Frangione B, Roses AD, et al. (1994) Apolipoprotein E associates with beta amyloid peptide of Alzheimer's disease to form novel monofibrils. Isoform apoE4 associates more efficiently than apoE3. J Clin Invest 94:860-869.

Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400:173-177.

Schenk D, Basi GS, Pangalos MN (2012) Treatment strategies targeting amyloid beta-protein. Cold Spring Harb Perspect Med 2:a006387.

Scherzer CR, Offe K, Gearing M, Rees HD, Fang G, Heilman CJ, Schaller C, Bujo H, Levey AI, Lah JJ (2004) Loss of apolipoprotein E receptor LR11 in Alzheimer disease. Arch Neurol 61:1200-1205.

Schroeder A, Fahrenholz F, Schmitt U (2009) Effect of a dominant-negative form of ADAM10 in a mouse model of Alzheimer's disease. J Alzheimers Dis 16:309-314.

Schroeter H, Bahia P, Spencer JPE, Sheppard O, Rattray M, Cadenas E, Rice-Evans C, Williams RJ (2007) (-)Epicatechin stimulates ERK-dependent cyclic AMP response element activity and up-regulates GluR2 in cortical neurons. Journal of Neurochemistry 101:1596-1606.

Schroeter H, Boyd C, Spencer JP, Williams RJ, Cadenas E, Rice-Evans C (2002) MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide. Neurobiol Aging 23:861-880.

Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M (2006) (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci U S A 103:1024-1029.

Schroeter H, Heiss C, Spencer JP, Keen CL, Lupton JR, Schmitz HH (2010) Recommending flavanols and procyanidins for cardiovascular health: current knowledge and future needs. Mol Aspects Med 31:546-557.

Schroeter H, Spencer JP, Rice-Evans C, Williams RJ (2001) Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. Biochem J 358:547-557.

Seger R, Ahn NG, Posada J, Munar ES, Jensen AM, Cooper JA, Cobb MH, Krebs EG (1992) Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. J Biol Chem 267:14373-14381.

Seguin J, Brulle L, Boyer R, Lu YM, Ramos Romano M, Touil YS, Scherman D, Bessodes M, Mignet N, Chabot GG (2013) Liposomal encapsulation of the natural flavonoid fisetin improves bioavailability and antitumor efficacy. International journal of pharmaceutics 444:146-154.

Selkoe DJ (1991a) Amyloid protein and Alzheimer's disease. Sci Am 265:68-71, 74-66, 78.

Selkoe DJ (1991b) The molecular pathology of Alzheimer's disease. Neuron 6:487-498.

Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. Science 298:789-791.

Selkoe DJ, Abraham CR, Podlisny MB, Duffy LK (1986) Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease. J Neurochem 46:1820-1834.

Selkoe DJ, Podlisny MB (2002) Deciphering the genetic basis of Alzheimer's disease. Annu Rev Genomics Hum Genet 3:67-99.

Seltzer B, Zolnouni P, Nunez M, Goldman R, Kumar D, Ieni J, Richardson S (2004) Efficacy of donepezil in early-stage Alzheimer disease: a randomized placebo-controlled trial. Arch Neurol 61:1852-1856.

Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. Cold Spring Harb Perspect Med 1:a006189.

Setou M, Nakagawa T, Seog DH, Hirokawa N (2000) Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. Science 288:1796-1802.

Shen J, Bronson RT, Chen DF, Xia W, Selkoe DJ, Tonegawa S (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. Cell 89:629-639.

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HA, Haines JL, Perkicak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM, St George-Hyslop PH (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375:754-760.

Shimmyo Y, Kihara T, Akaike A, Niidome T, Sugimoto H (2008a) Epigallocatechin-3-gallate and curcumin suppress amyloid beta-induced beta-site APP cleaving enzyme-1 upregulation. Neuroreport 19:1329-1333.

Shimmyo Y, Kihara T, Akaike A, Niidome T, Sugimoto H (2008b) Flavonols and flavones as BACE-1 inhibitors: Structure–activity relationship in cell-free, cell-based and in silico studies reveal novel pharmacophore features. Biochimica et Biophysica Acta (BBA) - General Subjects 1780:819-825.

Shioi J, Pangalos MN, Ripellino JA, Vassilacopoulou D, Mytilineou C, Margolis RU, Robakis NK (1995) The Alzheimer amyloid precursor proteoglycan (appican) is present in brain and is produced by astrocytes but not by neurons in primary neural cultures. J Biol Chem 270:11839-11844.

Sinha S, Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, Doan M, Dovey HF, Frigon N, Hong J, Jacobson-Croak K, Jewett N, Keim P, Knops J, Lieberburg I, Power M, Tan H, Tatsuno G, Tung J, Schenk D, Seubert P, Suomensaari SM, Wang S, Walker D, Zhao J, McConlogue L, John V (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature 402:537-540.

Sisodia SS (1992a) Beta-amyloid precursor protein cleavage by a membrane-bound protease. Proc Natl Acad Sci U S A 89:6075-6079.

Sisodia SS (1992b) Secretion of the beta-amyloid precursor protein. Ann N Y Acad Sci 674:53-57.

Sisodia SS, St George-Hyslop PH (2002) γ-Secretase, Notch, Aβ and Alzheimer's Disease: Where Do the Presenilins Fit In? Nature Reviews Neuroscience 3:281-290.

Slack BE, Ma LK, Seah CC (2001) Constitutive shedding of the amyloid precursor protein ectodomain is up-regulated by tumour necrosis factor-alpha converting enzyme. Biochem J 357:787-794.

Small SA, Gandy S (2006) Sorting through the cell biology of Alzheimer's disease: intracellular pathways to pathogenesis. Neuron 52:15-31.

Smith MA, Nunomura A, Zhu X, Takeda A, Perry G (2000) Metabolic, metallic, and mitotic sources of oxidative stress in Alzheimer disease. Antioxidants & redox signaling 2:413-420.

Soba P, Eggert S, Wagner K, Zentgraf H, Siehl K, Kreger S, Lower A, Langer A, Merdes G, Paro R, Masters CL, Muller U, Kins S, Beyreuther K (2005) Homo- and heterodimerization of APP family members promotes intercellular adhesion. EMBO J 24:3624-3634.

Spencer JP, Rice-Evans C, Williams RJ (2003) Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability. J Biol Chem 278:34783-34793.

Spencer JP, Schroeter H, Rechner AR, Rice-Evans C (2001) Bioavailability of flavan-3-ols and procyanidins: gastrointestinal tract influences and their relevance to bioactive forms in vivo. Antioxidants & redox signaling 3:1023-1039.

Spencer JPE (2010) The impact of fruit flavonoids on memory and cognition. British Journal of Nutrition 104:S40-S47.

Spillantini MG, Bird TD, Ghetti B (1998) Frontotemporal dementia and Parkinsonism linked to chromosome 17: a new group of tauopathies. Brain Pathol 8:387-402.

St George-Hyslop P, Fraser PE (2012) Assembly of the presenilin gamma-/epsilon-secretase complex. J Neurochem 120 Suppl 1:84-88.

St George-Hyslop P, Schmitt-Ulms G (2010) Alzheimer's disease: Selectively tuning gamma-secretase. Nature 467:36-37.

Steinbach JP, Muller U, Leist M, Li ZW, Nicotera P, Aguzzi A (1998) Hypersensitivity to seizures in beta-amyloid precursor protein deficient mice. Cell Death Differ 5:858-866.

Strittmatter WJ, Weisgraber KH, Goedert M, Saunders AM, Huang D, Corder EH, Dong LM, Jakes R, Alberts MJ, Gilbert JR, et al. (1994) Hypothesis: microtubule instability and paired helical filament formation in the Alzheimer disease brain are related to apolipoprotein E genotype. Experimental neurology 125:163-171; discussion 172-164.

Strittmatter WJ, Weisgraber KH, Huang DY, Dong LM, Salvesen GS, Pericak-Vance M, Schmechel D, Saunders AM, Goldgaber D, Roses AD (1993) Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. Proc Natl Acad Sci U S A 90:8098-8102.

Strohmeyer R, Shen Y, Rogers J (2000) Detection of complement alternative pathway mRNA and proteins in the Alzheimer's disease brain. Brain Res Mol Brain Res 81:7-18.

Suh J, Choi SH, Romano DM, Gannon MA, Lesinski AN, Kim DY, Tanzi RE (2013) ADAM10 Missense Mutations Potentiate beta-Amyloid Accumulation by Impairing Prodomain Chaperone Function. Neuron 80:385-401.

Suzuki K, Hayashi Y, Nakahara S, Kumazaki H, Prox J, Horiuchi K, Zeng M, Tanimura S, Nishiyama Y, Osawa S, Sehara-Fujisawa A, Saftig P, Yokoshima S, Fukuyama T, Matsuki N, Koyama R, Tomita T, Iwatsubo T (2012) Activity-dependent proteolytic cleavage of neuroligin-1. Neuron 76:410-422.

Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Jr., Eckman C, Golde TE, Younkin SG (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264:1336-1340.

Suzuki T, Nakaya T (2008) Regulation of amyloid beta-protein precursor by phosphorylation and protein interactions. J Biol Chem 283:29633-29637.

Sweatt JD (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol 14:311-317.

Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, Thinakaran G, Iwatsubo T (2003) The role of presenilin cofactors in the gamma-secretase complex. Nature 422:438-441.

Tamagno E, Bardini P, Obbili A, Vitali A, Borghi R, Zaccheo D, Pronzato MA, Danni O, Smith MA, Perry G, Tabaton M (2002) Oxidative stress increases expression and activity of BACE in NT2 neurons. Neurobiol Dis 10:279-288.

Tamagno E, Guglielmotto M, Aragno M, Borghi R, Autelli R, Giliberto L, Muraca G, Danni O, Zhu X, Smith MA, Perry G, Jo DG, Mattson MP, Tabaton M (2008) Oxidative stress activates a positive feedback between the gamma- and beta-secretase cleavages of the beta-amyloid precursor protein. J Neurochem 104:683-695.

Tamagno E, Guglielmotto M, Giliberto L, Vitali A, Borghi R, Autelli R, Danni O, Tabaton M (2009) JNK and ERK1/2 pathways have a dual opposite effect on the expression of BACE1. Neurobiol Aging 30:1563-1573.

Tamaoka A, Odaka A, Ishibashi Y, Usami M, Sahara N, Suzuki N, Nukina N, Mizusawa H, Shoji S, Kanazawa I, et al. (1994) APP717 missense mutation affects the ratio of amyloid beta protein species (A beta 1-42/43 and a beta 1-40) in familial Alzheimer's disease brain. J Biol Chem 269:32721-32724.

Tan JL, Li QX, Ciccotosto GD, Crouch PJ, Culvenor JG, White AR, Evin G (2013) Correction: Mild Oxidative Stress Induces Redistribution of BACE1 in Non-Apoptotic Conditions and Promotes the Amyloidogenic Processing of Alzheimer's Disease Amyloid Precursor Protein. PLoS ONE 8.

Tanaka S, Shiojiri S, Takahashi Y, Kitaguchi N, Ito H, Kameyama M, Kimura J, Nakamura S, Ueda K (1989) Tissue-specific expression of three types of beta-protein precursor mRNA: enhancement of protease inhibitor-harboring types in Alzheimer's disease brain. Biochem Biophys Res Commun 165:1406-1414.

Tanzi RE (2013) A brief history of Alzheimer's disease gene discovery. J Alzheimers Dis 33 Suppl 1:S5-13.

Tanzi RE, McClatchey AI, Lamperti ED, Villa-Komaroff L, Gusella JF, Neve RL (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. Nature 331:528-530.

Taru H, Iijima K, Hase M, Kirino Y, Yagi Y, Suzuki T (2002) Interaction of Alzheimer's beta -amyloid precursor family proteins with scaffold proteins of the JNK signaling cascade. J Biol Chem 277:20070-20078.

Taylor CJ, Ireland DR, Ballagh I, Bourne K, Marechal NM, Turner PR, Bilkey DK, Tate WP, Abraham WC (2008) Endogenous secreted amyloid precursor protein-alpha regulates hippocampal NMDA receptor function, long-term potentiation and spatial memory. Neurobiol Dis 31:250-260.

Thal DR, Rub U, Orantes M, Braak H (2002) Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology 58:1791-1800.

Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, Kim G, Ratovitsky T, Davenport F, Nordstedt C, Seeger M, Hardy J, Levey AI, Gandy SE, Jenkins NA, Copeland NG, Price DL, Sisodia SS (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. Neuron 17:181-190.

Thinakaran G, Harris CL, Ratovitski T, Davenport F, Slunt HH, Price DL, Borchelt DR, Sisodia SS (1997) Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. J Biol Chem 272:28415-28422.

Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5:173-183.

Thompson PM, Hayashi KM, de Zubicaray G, Janke AL, Rose SE, Semple J, Herman D, Hong MS, Dittmer SS, Doddrell DM, Toga AW (2003) Dynamics of gray matter loss in Alzheimer's disease. J Neurosci 23:994-1005.

Tian L, Wu X, Chi C, Han M, Xu T, Zhuang Y (2008) ADAM10 is essential for proteolytic activation of Notch during thymocyte development. International immunology 20:1181-1187.

To MD, Gokgoz N, Doyle TG, Donoviel DB, Knight JA, Hyslop PS, Bernstein A, Andrulis IL (2006) Functional characterization of novel presenilin-2 variants identified in human breast cancers. Oncogene 25:3557-3564.

Tolia A, Chavez-Gutierrez L, De Strooper B (2006) Contribution of presenilin transmembrane domains 6 and 7 to a water-containing cavity in the gamma-secretase complex. J Biol Chem 281:27633-27642.

Tsang SW, Lai MK, Kirvell S, Francis PT, Esiri MM, Hope T, Chen CP, Wong PT (2006) Impaired coupling of muscarinic M1 receptors to G-proteins in the

neocortex is associated with severity of dementia in Alzheimer's disease. Neurobiol Aging 27:1216-1223.

Turner AJ, Nalivaeva NN (2006) Proteinase dysbalance in pathology: the neprilysin (NEP) and angiotensin-converting enzyme (ACE) families. Cell Mol Biol (Noisy-le-grand) 52:40-48.

Van Broeckhoven C, Haan J, Bakker E, Hardy JA, Van Hul W, Wehnert A, Vegter-Van der Vlis M, Roos RA (1990) Amyloid beta protein precursor gene and hereditary cerebral hemorrhage with amyloidosis (Dutch). Science 248:1120-1122.

van Goor H, Melenhorst WB, Turner AJ, Holgate ST (2009) Adamalysins in biology and disease. The Journal of pathology 219:277-286.

Van Nostrand WE, Melchor JP, Cho HS, Greenberg SM, Rebeck GW (2001) Pathogenic effects of D23N Iowa mutant amyloid beta -protein. J Biol Chem 276:32860-32866.

van Praag H, Lucero MJ, Yeo GW, Stecker K, Heivand N, Zhao C, Yip E, Afanador M, Schroeter H, Hammerstone J, Gage FH (2007) Plant-Derived Flavanol (-)Epicatechin Enhances Angiogenesis and Retention of Spatial Memory in Mice. Journal of Neuroscience 27:5869-5878.

Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis J-C, Collins F, Treanor J, Rogers G, Citron M (1999) β-Secretase Cleavage of Alzheimer's Amyloid Precursor Protein by the Transmembrane Aspartic Protease BACE. Science 286:735-741.

Vassar R, Kovacs DM, Yan R, Wong PC (2009) The -Secretase Enzyme BACE in Health and Alzheimer's Disease: Regulation, Cell Biology, Function, and Therapeutic Potential. Journal of Neuroscience 29:12787-12794.

Vauzour D, Ravaioli G, Vafeiadou K, Rodriguez-Mateos A, Angeloni C, Spencer JP (2008) Peroxynitrite induced formation of the neurotoxins 5-S-cysteinyl-dopamine and DHBT-1: implications for Parkinson's disease and protection by polyphenols. Arch Biochem Biophys 476:145-151.

Vauzour D, Vafeiadou K, Rice-Evans C, Williams RJ, Spencer JP (2007) Activation of pro-survival Akt and ERK1/2 signalling pathways underlie the anti-apoptotic effects of flavanones in cortical neurons. J Neurochem 103:1355-1367.

Velliquette RA, O'Connor T, Vassar R (2005) Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. J Neurosci 25:10874-10883.

Verges DK, Restivo JL, Goebel WD, Holtzman DM, Cirrito JR (2011) Opposing synaptic regulation of amyloid-beta metabolism by NMDA receptors in vivo. J Neurosci 31:11328-11337.

Vingtdeux V, Marambaud P (2012) Identification and biology of alpha-secretase. J Neurochem 120 Suppl 1:34-45.

Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269:5241-5248.

von Koch CS, Zheng H, Chen H, Trumbauer M, Thinakaran G, van der Ploeg LH, Price DL, Sisodia SS (1997) Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. Neurobiol Aging 18:661-669.

Wakabayashi T, De Strooper B (2008) Presenilins: members of the gamma-secretase quartets, but part-time soloists too. Physiology (Bethesda) 23:194-204.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid [beta] protein potently inhibit hippocampal long-term potentiation in vivo. Nature 416:535-539.

Walsh DM, Selkoe DJ (2007) A? Oligomers ? a decade of discovery. Journal of Neurochemistry 101:1172-1184.

Wan XZ, Li B, Li YC, Yang XL, Zhang W, Zhong L, Tang SJ (2012) Activation of NMDA receptors upregulates a disintegrin and metalloproteinase 10 via a Wnt/MAPK signaling pathway. J Neurosci 32:3910-3916.

Wang GP, Grundke-Iqbal I, Kascsak RJ, Iqbal K, Wisniewski HM (1984) Alzheimer neurofibrillary tangles: monoclonal antibodies to inherent antigen(s). Acta Neuropathol 62:268-275.

Wang H, Li R, Shen Y (2013) beta-Secretase: its biology as a therapeutic target in diseases. Trends in pharmacological sciences 34:215-225.

Wang J, Ferruzzi MG, Ho L, Blount J, Janle EM, Gong B, Pan Y, Gowda GA, Raftery D, Arrieta-Cruz I, Sharma V, Cooper B, Lobo J, Simon JE, Zhang C, Cheng A, Qian X, Ono K, Teplow DB, Pavlides C, Dixon RA, Pasinetti GM (2012) Brain-Targeted Proanthocyanidin Metabolites for Alzheimer's Disease Treatment. J Neurosci 32:5144-5150.

Wang J, Ho L, Zhao W, Ono K, Rosensweig C, Chen L, Humala N, Teplow DB, Pasinetti GM (2008) Grape-Derived Polyphenolics Prevent A Oligomerization and Attenuate Cognitive Deterioration in a Mouse Model of Alzheimer's Disease. Journal of Neuroscience 28:6388-6392.

Wang J, Ho L, Zhao Z, Seror I, Humala N, Dickstein DL, Thiyagarajan M, Percival SS, Talcott ST, Pasinetti GM (2006) Moderate consumption of Cabernet Sauvignon attenuates A neuropathology in a mouse model of Alzheimer's disease. The FASEB Journal 20:2313-2320.

Wang M, Wu L, Wang L, Xin X (2010) Down-regulation of Notch1 by gamma-secretase inhibition contributes to cell growth inhibition and apoptosis in ovarian cancer cells A2780. Biochem Biophys Res Commun 393:144-149.

Weller RO, Massey A, Kuo YM, Roher AE (2000) Cerebral amyloid angiopathy: accumulation of A beta in interstitial fluid drainage pathways in Alzheimer's disease. Ann N Y Acad Sci 903:110-117.

Weskamp G, Ford JW, Sturgill J, Martin S, Docherty AJ, Swendeman S, Broadway N, Hartmann D, Saftig P, Umland S, Sehara-Fujisawa A, Black RA, Ludwig A, Becherer JD, Conrad DH, Blobel CP (2006) ADAM10 is a principal 'sheddase' of the low-affinity immunoglobulin E receptor CD23. Nature immunology 7:1293-1298.

Weston LA, Mathesius U (2013) Flavonoids: their structure, biosynthesis and role in the rhizosphere, including allelopathy. Journal of chemical ecology 39:283-297.

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237-1239.

Wilcock GK, Lilienfeld S, Gaens E (2000) Efficacy and safety of galantamine in patients with mild to moderate Alzheimer's disease: multicentre randomised controlled trial. Galantamine International-1 Study Group. BMJ 321:1445-1449.

Williams RJ, Spencer JP (2012) Flavonoids, cognition, and dementia: Actions, mechanisms, and potential therapeutic utility for Alzheimer disease. Free Radic Biol Med 52:35-45.

Willnow TE, Andersen OM (2013) Sorting receptor SORLA - a trafficking path to avoid Alzheimer disease. J Cell Sci 126:2751-2760.

Wimo A, Jonsson L, Bond J, Prince M, Winblad B (2013) The worldwide economic impact of dementia 2010. Alzheimers Dement 9:1-11 e13.

Winblad B, Poritis N (1999) Memantine in severe dementia: results of the 9M-Best Study (Benefit and efficacy in severely demented patients during treatment with memantine). Int J Geriatr Psychiatry 14:135-146.

Wolfe MS, Guenette SY (2007) APP at a glance. Journal of Cell Science 120:3157-3161.

Wong PC, Zheng H, Chen H, Becher MW, Sirinathsinghji DJ, Trumbauer ME, Chen HY, Price DL, Van der Ploeg LH, Sisodia SS (1997) Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. Nature 387:288-292.

Wu HY, Hudry E, Hashimoto T, Kuchibhotla K, Rozkalne A, Fan Z, Spires-Jones T, Xie H, Arbel-Ornath M, Grosskreutz CL, Bacskai BJ, Hyman BT (2010) Amyloid Induces the Morphological Neurodegenerative Triad of Spine Loss,

Dendritic Simplification, and Neuritic Dystrophies through Calcineurin Activation. Journal of Neuroscience 30:2636-2649.

Yan R, Bienkowski MJ, Shuck ME, Miao H, Tory MC, Pauley AM, Brashier JR, Stratman NC, Mathews WR, Buhl AE, Carter DB, Tomasselli AG, Parodi LA, Heinrikson RL, Gurney ME (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature 402:533-537.

Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, Chen PP, Kayed R, Glabe CG, Frautschy SA, Cole GM (2005) Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. J Biol Chem 280:5892-5901.

Yang LB, Lindholm K, Yan R, Citron M, Xia W, Yang XL, Beach T, Sue L, Wong P, Price D, Li R, Shen Y (2003) Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. Nat Med 9:3-4.

Yang Z (2006) A Dominant Role for FE65 (APBB1) in Nuclear Signaling. Journal of Biological Chemistry 281:4207-4214.

Yankner BA, Duffy LK, Kirschner DA (1990) Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. Science 250:279-282.

Ying SW, Futter M, Rosenblum K, Webber MJ, Hunt SP, Bliss TV, Bramham CR (2002) Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. J Neurosci 22:1532-1540.

Yoshikai S, Sasaki H, Doh-ura K, Furuya H, Sakaki Y (1991) Genomic organization of the human-amyloid beta-protein precursor gene. Gene 102:291-292.

Yu G, Chen F, Nishimura M, Steiner H, Tandon A, Kawarai T, Arawaka S, Supala A, Song YQ, Rogaeva E, Holmes E, Zhang DM, Milman P, Fraser P, Haass C, St George-Hyslop P (2000a) Mutation of conserved aspartates affect maturation of presenilin 1 and presenilin 2 complexes. Acta Neurol Scand Suppl 176:6-11.

Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T, Supala A, Levesque L, Yu H, Yang DS, Holmes E, Milman P, Liang Y, Zhang DM, Xu DH, Sato C, Rogaev E, Smith M, Janus C, Zhang Y, Aebersold R, Farrer LS, Sorbi S, Bruni A, Fraser P, St George-Hyslop P (2000b) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature 407:48-54.

Zhang X, Zhou K, Wang R, Cui J, Lipton SA, Liao FF, Xu H, Zhang YW (2007) Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia increases BACE1 expression and beta-amyloid generation. J Biol Chem 282:10873-10880.

Zhang YQ, Sarge KD (2008) Sumoylation of amyloid precursor protein negatively regulates Abeta aggregate levels. Biochem Biophys Res Commun 374:673-678.

Zheng H, Jiang M, Trumbauer ME, Sirinathsinghji DJ, Hopkins R, Smith DW, Heavens RP, Dawson GR, Boyce S, Conner MW, Stevens KA, Slunt HH, Sisoda SS, Chen HY, Van der Ploeg LH (1995) beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81:525-531.

Zheng H, Koo EH (2011) Biology and pathophysiology of the amyloid precursor protein. Mol Neurodegener 6:27.

Zhou L, Brouwers N, Benilova I, Vandersteen A, Mercken M, Van Laere K, Van Damme P, Demedts D, Van Leuven F, Sleegers K, Broersen K, Van Broeckhoven C, Vandenberghe R, De Strooper B (2011) Amyloid precursor protein mutation E682K at the alternative beta-secretase cleavage beta'-site increases Abeta generation. EMBO Mol Med 3:291-302.

Zhu JJ, Qin Y, Zhao M, Van Aelst L, Malinow R (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. Cell 110:443-455.

Zweig RM, Ross CA, Hedreen JC, Steele C, Cardillo JE, Whitehouse PJ, Folstein MF, Price DL (1988) The neuropathology of aminergic nuclei in Alzheimer's disease. Ann Neurol 24:233-242.